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Phenotypic divergence of the three-spined stickleback
(*Gasterosteus aculeatus*) – Relating body shape and habitat

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1. Abstract - Zusammenfassung - Resumé

1.1 Abstract

Environmental factors as e.g. temperature, salinity and the like are known to influence animal development via phenotypic plasticity and to be a driving force behind adaptive evolution. The three-spined stickleback (*Gasterosteus aculeatus*) is known to occur in three ecotypes (marine, anadromous and freshwater) and various morphological forms. Each of the ecotypes has been found to be specifically adapted to their particular habitats, especially with respect to life cycle, swimming physiology, feeding ecology and behaviour. The marine ecotype is the most ancestral form from which the anadromous and further the derived freshwater ecotype have evolved several times independently. Changes in body shape emerge very fast in three-spined stickleback populations introduced into a new environment. This study focuses on the Wadden Sea of Southern Denmark and Northern Germany as well as some upcountry areas in Southern Denmark. One marine, one freshwater and three anadromous populations were analysed. The body shape changes were analysed using geometric morphometrics. This study is based on a very large set of landmarks (45 lms), thus very detailed results could be achieved. In all populations, regardless which ecotype they belong to, a strong sexual dimorphism was found. Sexual dimorphism in body shape is likely to reflect adaptations to different reproductive roles and different ecological niches occupied by the two sexes. The males are more benthic living, while the females inhabit more open waters. The comparison of ecotypes revealed a clear difference in body shape between the marine and the anadromous ecotype. These two ecotypes, which are often lumped together in the literature, should therefore be treated separately in future studies. Further, this study showed a continuous trend from the marine to the anadromous and the freshwater ecotype in the following traits: the size of the eye is increasing, the body depth and the size of the ectocoracoid are decreasing and the ventral origin of the pectoral fin is shifted in a more anterior position. The anadromous ecotype has been confirmed as an intermediate state between the marine and the freshwater ecotype.

Keywords: Phenotypic divergence, *Gasterosteus aculeatus*, ecotype, body shape, sexual dimorphism, geometric morphometrics, evolutionary diversification

1.2 Zusammenfassung

Es ist bekannt, dass Umweltfaktoren, wie z.B. Temperatur, Salinität und ähnliche, Einfluss auf die Entwicklung bei Tieren via phänotypischer Plastizität haben und die treibende Kraft hinter der adaptiven Evolution sind. Der Dreistachlige Stichling (*Gasterosteus aculeatus*) ist in drei Ökotypen (marin, anadrom und Süßwasser lebend) und einer Vielzahl von morphologischen Formen bekannt. Jeder der drei Ökotypen hat spezielle Anpassungen an den jeweiligen Lebensraum, vor allem im Bezug auf ihren Lebenszyklus, ihre Schwimmphysiologie, ihre Fressökologie und ihr Fressverhalten. Der marine Ökotyp ist der Ursprünglichste aus dem sich der anadrome und der abgeleitete Süßwasser lebende Ökotyp entwickelt haben. Veränderungen in der Körperform treten bei Populationen des Dreistachligen Stichlings innerhalb kurzer Zeit auf, nachdem sie einen neuen Lebensraum besiedelt haben. Diese Studie wurde an den Wattenmeerküsten Dänemarks und Norddeutschlands sowie im Landesinneren Süddänemarks durchgeführt. Insgesamt wurden eine marin, eine Süßwasser und drei anadrom lebende Populationen untersucht. Die Analyse der Körperformunterschiede wurde mittels Geometrischer Morphometrie durchgeführt. Die detaillierten Resultate dieser Studie wurden durch die sehr hohe Anzahl an verwendeten „landmarks“ (45 lms) erreicht. In allen untersuchten Populationen, egal welchem Ökotypus sie angehören, wurde ein starker Sexualdimorphismus festgestellt. Der Sexualdimorphismus in der Körperform spiegelt möglicherweise eine Anpassung an die unterschiedlichen reproduktiven Rollen und die unterschiedlichen ökologischen Nischen, die die beiden Geschlechter nutzen, wider. Die Männchen sind benthisch lebend, während sich die Weibchen vermehrt in der freien Wassersäule aufhalten. Der Vergleich der Ökotypen ergab klare Unterschiede zwischen der Körperform des marinen und des anadromen Ökotypus. Daher sollten diese zwei Ökotypen, die in der Literatur oft in einen Topf geworfen werden, in kommenden Studien als getrennte Ökotypen behandelt werden. Weiters zeigen die Resultate dieser Studie einen durchgehenden Trend in den folgenden morphologischen Merkmalen vom marinen über den anadromen zum Süßwasser lebenden Ökotypus hin: Das Auge wird größer, die Körperhöhe sowie die Größe des Ectocoracoides nehmen ab und der ventrale Ursprung der Pectoralis ist anteriorer. Der Status des anadromen Ökotypus als Übergangsform zwischen dem marinen und dem Süßwasser Ökotypus wurde bestätigt.

Schlüsselwörter: Phänotypische Divergenz, *Gasterosteus aculeatus*, Ökotyp, Körperform, Sexualdimorphismus, Geometrische Morphometrie, Evolutionäre Diversifikation

1.3 Resumé

Miljøfaktorer som f.eks. temperatur, salinitet og lignende har indflydelse på dyrenes udvikling via fænotypisk plasticitet og er en drivende kraft bag adaptiv evolution. Den trepiggede hundestejle (*Gasterosteus aculeatus*) forekommer i tre forskellige økoter (marin, anadrom og ferskvand) og mange morfologiske former. Hver økotype udviser tilpasninger til specifikke miljøforhold i blandt andet svømmefysiologi, fødeøkologi, adfærd og livscyklus. Den anadrome økotype har udviklet sig fra den oprindelige marine økotype. Ferskvandsøkotypen har sekundært udviklet sig fra den anadrome økotype. Forandringer i kropsform har vist sig at opstå i løbet af relativt kort tid efter at en population af trepigget hundestejle er indvandret til et nyt miljø. Denne undersøgelse blev foretaget i det danske og nordtyske Vadehav og i den syddanske region. I alt blev en ferskvandspopulation, en marin og tre anadrome populationer undersøgt. Analysen af kropsformsforskellene blev gennemført ved brug af geometrisk morfometri. De detaljerede resultater af denne undersøgelse blev opnået ved hjælp af det store antal af "landmarks" (45 lms) anvendt i denne studie. En meget stærk seksuel dimorfisme blev fundet i alle undersøgte populationer, uafhængigt af hvilken økotype de tilhørte. Den seksuelle dimorfisme i kropsformen er muligvis en tilpasning til de forskellige reproduktive roller og de forskellige økologiske nicher, de to køn udnytter. Hannerne lever mere bentisk, hvorimod hunnerne opholder sig mere i det åbne vand. Sammenligningen af økoterne påviste en klar forskel i kropsformen mellem den marine og den anadrome økotype. På grund af denne forskel i kropsformen bør disse to økoter, som i litteraturen ofte bliver blandet sammen, betragtes særskilt i kommende undersøgelser. Resultaterne af dette studie viser en tydelig trend fra den marine over den anadrome til ferskvandsøkotypen ved følgende morfologiske kendetegn: øjet bliver større, kropshøjden og størrelsen af ectocoracoiden bliver mindre og det ventrale udspring af den pectorale finne er forskudt i anterior retning. Desuden bekræftede undersøgelsen, at den anadrome økotype er et overgangsstadium mellem den marine økotype og ferskvandsøkotypen.

Nøgleord: Fænotypisk divergens, *Gasterosteus aculeatus*, økotype, kropsform, seksuel dimorfisme, geometrisk morfometri, evolutionær diversifikation

2. Introduction

The three-spined stickleback (*Gasterosteus aculeatus*) is an up to 110 mm long euryhaline fish (Bell and Foster, 1994b). Already Bell and Foster (1994b) mentioned that the fact that every attempt to characterise the three-spined stickleback briefly has to fail, makes it so fascinating. In contemporary biology it is a common model organism for studies on phenotypic plasticity, adaptive radiation and parallel evolution (e.g. Bell and Foster, 1994b; Schluter, 2000; Colosimo *et al.*, 2005; Östlund-Nilsson *et al.*, 2007; Spoljaric and Reimchen, 2011). Important characteristics of *G. aculeatus* for these studies are the large phenotypic variability, the adaption to many different environments, a fast succession of generations (e.g. Wootton, 1984; Bell and Foster, 1994b; Paepke, 2002) and it is easy to keep in tanks for experimental studies.

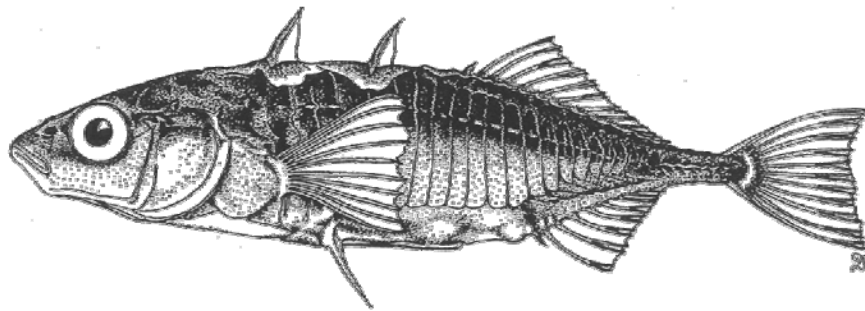


Fig. 1. An adult specimen of the three-spined stickleback (*Gasterosteus aculeatus*); (Wootton, 1984).

2.1 Distribution and ecotypes

Gasterosteus aculeatus is distributed on all three Northern continents (Wootton, 1984). The distribution in marine waters ranges from the coasts of the White Sea, along the Atlantic, North Sea and Baltic Sea coasts to the Iberian Peninsula and the coasts of the Black Sea. There are no recent marine populations in the Mediterranean Sea. Freshwater ecosystems are inhabited in near-shore river systems of Northern Europe (Scandinavia, Finland, Iceland, Russia, the Baltics and Poland), the British Isles, Western Europe, the tributaries from the Black Sea to the Baltic drainages and isolated populations in the coastal areas of the Mediterranean Sea (e.g. Wootton, 1984; Bell and Foster, 1994b; Paepke, 2002; Mäkinen *et al.*, 2006). The recent distribution of *G. aculeatus* in Europe is influenced by the last glacial maximum approximately 10 000 years ago (e.g. Münzing, 1963; Wootton, 2009). Mäkinen *et*

al. (2006) analysed the genetic structure of the three-spined stickleback in Europe and came up with six geographical genetic clusters. One of them, the North Sea cluster, covers the area investigated in this study.

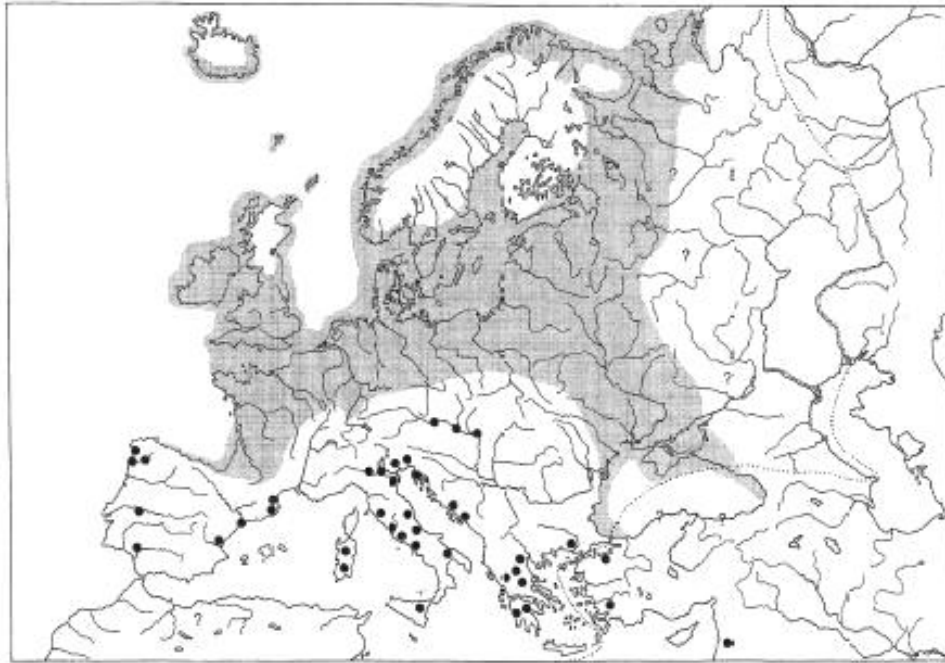


Fig. 2. The geographical distribution of *G. aculeatus* in Europe. Filled dots in western and southern Europe indicate the location of partially or fully isolated populations beyond the main area of the range. These include introduced populations in the upper Danube drainage area and Lake Constance; modified after Paepke (2002).

The three-spined stickleback is known in three ecological forms and various morphological forms (e.g. Wootton, 1984; 2009; Reimchen, 1994; Paepke, 2002). The first ecotype is the marine one, spending the entire life cycle in marine habitats. This ecotype spends most of the year in open oceans, returning to coastal habitats only in the breeding season (e.g. Wootton, 1984; Bell and Foster, 1994b; Paepke, 2002). *G. aculeatus* is a marine fish, hence this is the most ancestral ecotype from which the anadromous ecotype has evolved (Münzing, 1959; Paepke, 2002; Schluter and Conte, 2009; Wootton, 2009). The anadromous ecotype is characterised by a migrating life cycle. The adult specimens live in open marine waters until they reach sexual maturity. In spring they migrate into near-shore brackish or freshwater habitats for breeding. Most of the adult specimens die in the freshwaters after the breeding season. The juvenile three-spined sticklebacks spend their first weeks after hatching in the freshwater habitats (e.g. Wootton, 1984; Paepke, 2002) and start migrating downstream to marine waters at a standard length of approximately 18 mm (Münzing, 1959). These two ecotypes are in the literature often referred to as the marine or oceanic three-spined

sticklebacks, although they have completely different life histories. The third ecotype is the resident freshwater ecotype, living and breeding in freshwater habitats. These different habitats reach from streams and creeks to lakes of very different size (e.g. Wootton, 1984; 2009; Bell and Foster, 1994b; Paepke, 2002). On the one hand, Bell and Baumgartner (1984) reported three-spined sticklebacks in an extremely small pond of 0.026 ha, and on the other hand, Hubbs *et al.* (2004) report them in Lake Ontario with a size of 196 840 ha.

2.2 Evolution from marine to freshwater populations

Populations of the marine and the anadromous ecotype of the three-spined stickleback have colonised numerous freshwater habitats on the northern hemisphere after the last glacial maximum (e.g. Münzing, 1963; Klepaker, 1993; Wootton, 2009). The resident freshwater ecotype has then evolved several times independently, especially from the anadromous ecotype (e.g. Bell *et al.*, 2004; Colosimo *et al.*, 2005). Wootton (2009) predicted that this is only possible because *G. aculeatus* is an unusual teleost, referring to its geographic distribution and the range of habitats it can occupy. These colonisation events make the three-spined stickleback a good example how natural selection acts on closely-related organisms introduced into similar environments. Further they demonstrate that parallel evolution is one of the most notable outcomes of natural selection (Aguirre and Bell, 2012). Natural selection plays also a key role in adaptive radiation, which is another important phenomenon in evolutionary biology (Schluter, 2000). The postglacial radiation of the three-spined stickleback is one of the most enlightening cases of adaptive radiation (Aguirre *et al.*, 2008), because it is one of the seldom occasions where the ancestral properties are known. This enables the examination of the process right from the beginning, as studies such as Klepaker (1993) and Aguirre and Bell (2012) show. With their studies Klepaker (1993); Kristjansson (2005); Aguirre *et al.* (2008) and Aguirre and Bell (2012) provided insights in the short time spans needed for phenotypic diversity to evolve and in which directions this evolution may lead.

Leinonen *et al.* (2006; 2011b) working on European three-spined sticklebacks also found complex morphological changes associated with the transition from marine to freshwater life, as reduction in bony armour and changes in body shape. The latter is one of the central complex quantitative traits that is necessary to understand the evolutionary diversification, but these complex traits are hard to quantify (Aguirre and Bell, 2012).

It is well known that ecological parameters are strongly associated with phenotypic differentiation (e.g. Spoljaric and Reimchen, 2007; Hendry *et al.*, 2009). With the migration into a new environment many factors change, such as osmotic properties, nutrition, predation, competition, parasite infestation, habitat structure and available prey (e.g. Klepaker, 1993; Walker, 1997). Hence foraging and predator evasion influenced by the hydrodynamic factors can be assumed to affect body shape of fishes (Spoljaric and Reimchen, 2007). Thus the high phenotypic plasticity in *G. aculeatus* might be important for its adaptation to new environments (e.g. Wund *et al.*, 2008; Hendry *et al.*, 2009). Despite apparent phenotypic plasticity, body shape and other phenotypic traits are also genetically determined (e.g. Aguirre *et al.*, 2008; Aguirre, 2009; Schluter *et al.*, 2010; Leinonen *et al.*, 2011a). This has also been shown by Hendry *et al.* (2002) in their common-garden experiments. Colosimo *et al.* (2005) and Schluter and Conte (2009) found that marine and anadromous populations are a reservoir of certain allele variations favourable in freshwater habitats and available for selection when freshwater is inhabited.

2.3 Body shape

Fishes in general differ widely in body shape and often this corresponds to adaptations to different habitats (Webb, 1982). In the last decades *G. aculeatus* was studied extensively as reviewed by Wootton (2009). Mostly body shape variations between different sexes, ecotypes or habitats within freshwater were examined. In general two typical body shapes are described, which appear to be adaptations to swimming and feeding behaviour (e.g. Taylor and McPhail, 1986; Schluter and McPhail, 1992; Walker, 1997). The more limnetic body shape is characterised by a streamlined body shape, a long and narrow caudal peduncle, small eyes, a small mouth and a long snout. This body shape is an adaptation to long swimming distances in open waters, because it minimizes the drag (Taylor and McPhail, 1986; Walker, 1997). It is further favourable for feeding on zooplankton, which has a spatially heterogeneous distribution in open waters (e.g. Pinel-Alloul *et al.*, 1988; Downing, 1991; Folt and Schultze, 1993). The more benthic body shape is deeper-bodied, with a short and thick caudal peduncle, a large head, eye and mouth but a short snout (e.g. Walker, 1997; Wund *et al.*, 2008). This body shape is an adaptation for a better manoeuvrability in dense vegetation or structures and for feeding on benthic organisms (Walker, 1997). Common benthic prey includes for example chironomid larvae and amphipods (e.g. Hart and Gill,

1994; Wootton, 1994), preferring habitats with dense aquatic vegetation (e.g. Cyr and Downing, 1988; Downing, 1991). In some lakes sympatric populations of *G. aculeatus* occur, using the two different ecological niches explained above (e.g. Schluter and McPhail, 1992; Wund *et al.*, 2008; Wootton, 2009).

2.4 Sexual dimorphism

Knowledge about sexual dimorphism is crucial for the understanding of the ecology, behaviour and life history of a species, as well as the comparison of populations (Kitano *et al.*, 2007). The sexual dimorphism in three-spined sticklebacks is well known and studied in large parts of its distribution area, for example in North America (e.g. Schluter and McPhail, 1992; Reimchen and Nosil, 2006; Aguirre and Akinpelu, 2010), in Japan (Kitano *et al.*, 2007) and Europe (e.g. Kristjansson *et al.*, 2002a; Leinonen *et al.*, 2011a). The males and females of *G. aculeatus* have different reproductive roles (Wootton, 1984; Bell and Foster, 1994a). Beside this, body size and other morphological traits are important in three-spined sticklebacks' male and female mate choice. It has thus been suggested that the sexual dimorphism of these traits had evolved by sexual selection (e.g. Wootton, 1984; Schluter, 2001; McKinnon and Rundle, 2002). The fact that several sexually dimorphic traits of three-spined sticklebacks are associated with multiple ecological factors leads to the assumption that natural selection in different ecological niches also affects the evolution of the sexual dimorphism (Reimchen and Nosil, 2001; 2004). In three-spined sticklebacks the sex is genetically fixed (Peichel *et al.*, 2004). Kitano *et al.* (2007) suggest that sexual dimorphism, at least in body shape, is regulated by reproductive hormones and hence is a secondary sexual character because it was only significant after the specimens started breeding. Further the laboratory experiments pointed towards a genetic basis of sexual dimorphism in body shape. Kitano *et al.* (2007) concluded that further studies will be necessary to determine what genes or hormones in three-spined sticklebacks regulate the sexual dimorphism in body shape. Males and females are genetically similar in the basis of body shape and armour traits but this does not seem to limit the evolution of sexual dimorphism (Leinonen *et al.*, 2011a).

Very eye-catching is the breeding colour in males during breeding season, colouring the eye blue and the throat, cheek, opercle and breast red (Paepke, 2002), see chapter 3.1. In general females are reported to have a longer standard length (e.g. Reimchen and Nelson, 1987; Kitano *et al.*, 2007; Spoljaric and Reimchen, 2008). More subtle are the differences in

body shape and distinct morphological traits. Females have a larger pelvic girdle, a more anterior base of the pectoral fin and a larger region posterior to the eye. The males have a larger head (both in length and depth) and larger head structures like the mouth, the upper lip, the jaw and the snout. Further males have more posteriad dorsal spines, a deeper body, a longer dorsal fin and a longer anal fin (e.g. Kristjansson *et al.*, 2002a; Kitano *et al.*, 2007; Spoljaric and Reimchen, 2008; Aguirre and Akinpelu, 2010).

2.5 Why geometric morphometrics

Geometric morphometrics are a landmark based method, as explained in chapter 3.4. This method enables an efficient description of statistical results and due to the manifold possibilities of visualisation it is very effective in the presentation of explorative studies. Complex traits such as body shape are hard to study, but with a large set of landmarks it is possible to cover the overall body shape and not only single measurements as in traditional morphometrics. This provides detailed information on often subtle differences in specific body segments or traits.

2.6 Aims

This diploma thesis focuses on three-spined sticklebacks of the North Sea and near shore river systems in south Denmark. Since most studies take place in North America, one aim is the contribution of knowledge on European three-spined sticklebacks; especially Denmark is a white spot on the sticklebacks map. A particular focus lays on the association of microgeographical and phenotypic variation with the analyses of morphological divergence between the three different ecotypes. Important is the clear separation of marine and anadromous populations, which in literature often are treated as the same. Further the analysed freshwater population has been founded recently, allowing an insight in early stages of the adaptation to a new environment and the related changes in body shape. Another aim is determining the differences between males and females to clearly distinguish between shared and sex-specific characteristics. Compared with many other recent studies on body shape in three-spined sticklebacks, this one uses a larger landmark set providing detailed information of the body shape and specific traits, which are often not considered.

3. Material and methods

3.1 Sample

This diploma thesis is based on 5 three-spined stickleback (*Gasterosteus aculeatus*) populations belonging to three different ecotypes: marine, anadromous and freshwater. The sampling locations are described in section 3.2. The sampling was done in spring 2011. Between 100 and 300 three-spined sticklebacks were collected from each sampling site. The fish were euthanized with clove oil (one drop in one litre water). They were further fixed in 4% formaldehyde for four days. For fixing the anadromous and the freshwater populations, the used formaldehyde was watered down to 4% with freshwater; for the marine population only formaldehyde watered down with marine water was available. Subsequently, the fish were washed and placed for one hour in freshwater. Then the fish were passed through an alcohol series to wash out the formaldehyde and to avoid a fast shrinking of the tissue. During the complete alcohol series and the preservation the fish were stored vertically in glasses or single plastic tubes after labelling.

Tab. 1. Alcohol series for fixing the fish; d – days, h - hours.

| Time | Alcohol |
|--------------|---------|
| 4 h | 30% |
| 4 h | 30% |
| 24 h | 60% |
| 3 d | 70% |
| Preservation | 70% |

For this study only intact specimens without bending artefacts or visible deformations due to parasites were selected. The sexes were identified by the well known sexual dimorphism occurring during mating time, where the males' throat and breast becomes red and the eyes colour blue (Wootton, 1984), see Fig. 3. Due to these characters 32 adult females and 32 adult males from each population were chosen. After the shape analyses the fish were dissected in order to determine the sex by the gonads. This led to the actual specimen numbers for both sexes presented in Tab. 2. In the same step the specimens were checked for parasites. Only in the freshwater population (UPF) *Schistocephalus solidus* infected specimens were found. Due to the possible influence of the parasite on the body shape all parasitized specimens (six females and thirteen males) were excluded. Additionally, four

females and two males of the marine population (SPK) had to be excluded for the shape analyses because of fixation artefacts. Tab. 2 shows the final number, sex distribution, and the males' standard length (SL) range of the analysed individuals.



Fig. 3. Image of the head of a male three-spined stickleback in breeding colour (blue eye; red throat, cheek, opercle and breast).

Tab. 2. List of the populations, the population abbreviations (Abbr.), the ecotype they belong to, and the number of females and males for each population analysed in this study with the minimum (min) and maximum (max) standard length (SL) for the males (m) of each population.

| Population | Abbr. | Ecotype | Number females | Number males | Min SL m (mm) | Max SL m (mm) |
|---|-------|------------|----------------|--------------|---------------|---------------|
| Brede Å system Kisbæk | BKD | Anadromous | 18 | 46 | 44.43 | 57.48 |
| Ditch to Ho bight south of Sønderballe | HSS | Anadromous | 30 | 34 | 47.90 | 57.22 |
| Rømø, Juvre “put&take” fishpond | RJF | Anadromous | 32 | 32 | 41.20 | 58.91 |
| Sylt, Königshafen tidal creek | SPK | Marine | 26 | 32 | 47.91 | 56.50 |
| Uge “put&take” fishpond | UPF | Freshwater | 24 | 21 | 41.30 | 51.34 |

For the analyses of sexual dimorphism all specimens given in Tab. 2 were used. For the ecotype differences only the males were examined because the populations were sampled in a time span of three month. This leads to differences in egg maturity in the female specimens, influencing the body shape and further the results.

3.2 Sampling sites

The three-spined stickleback populations were sampled in the Wadden Sea (North Sea) of south-western Denmark and northern Germany and at two study sites in upcountry Denmark (Fig. 4).

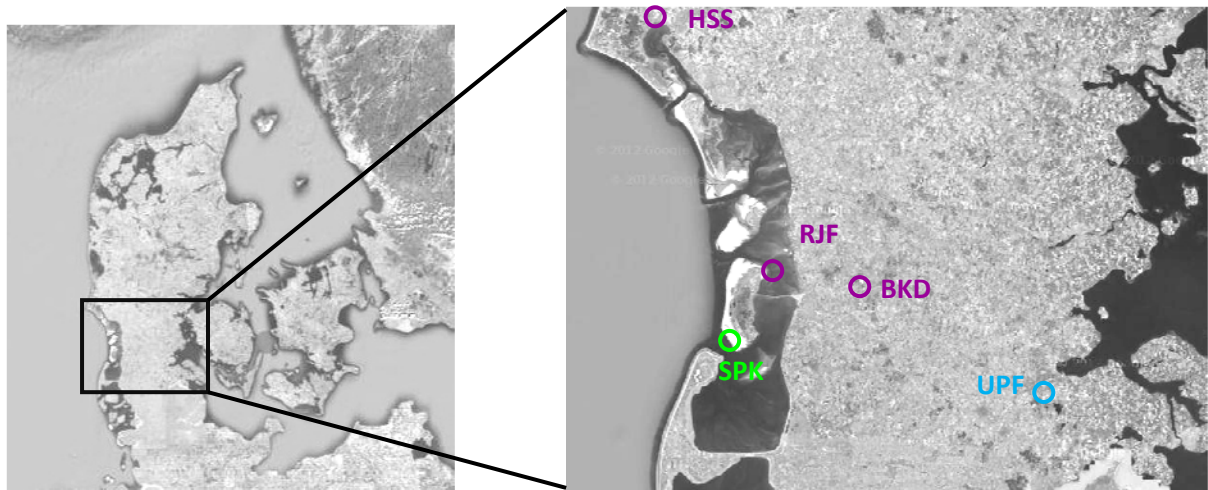


Fig. 4. Sampling sites in Denmark and Germany, purple – anadromous populations, green – marine population, blue – freshwater population. For population abbreviations see Tab. 2 (modified after maps.google.at).

The marine population (SPK) (Fig. 4) was sampled in a tidal creek in the bight of Königshafen, Sylt in northern Germany with a salinity of 27.8 ‰ at the sampling time. The sampling was done in April 2011, in the most upcountry end of the tidal creek, where it expands from 0.3 m to 4.5 m width, see Fig. 5a. In the shallow part, with a water depth of approximately 0.1 m – 0.6 m the fish were caught with dip nets and in the deeper areas with a water depth of up to 1.2 m with a seine. The vegetation in the sampling area includes some patches of dwarf eelgrass (*Zostera noltii*) and regions with undefined algae on the sand surface (Fig. 5b). Three-spined sticklebacks use *Zostera noltii* beds in the bight of Königshafen as spawning areas (Polte and Asmus, 2006). In winter the temperature in the near-shore shallow areas in the bight is too low and the deeper tidal creeks have a too strong current velocity forcing the threespine sticklebacks to migrate into deeper, open water areas of the North Sea.

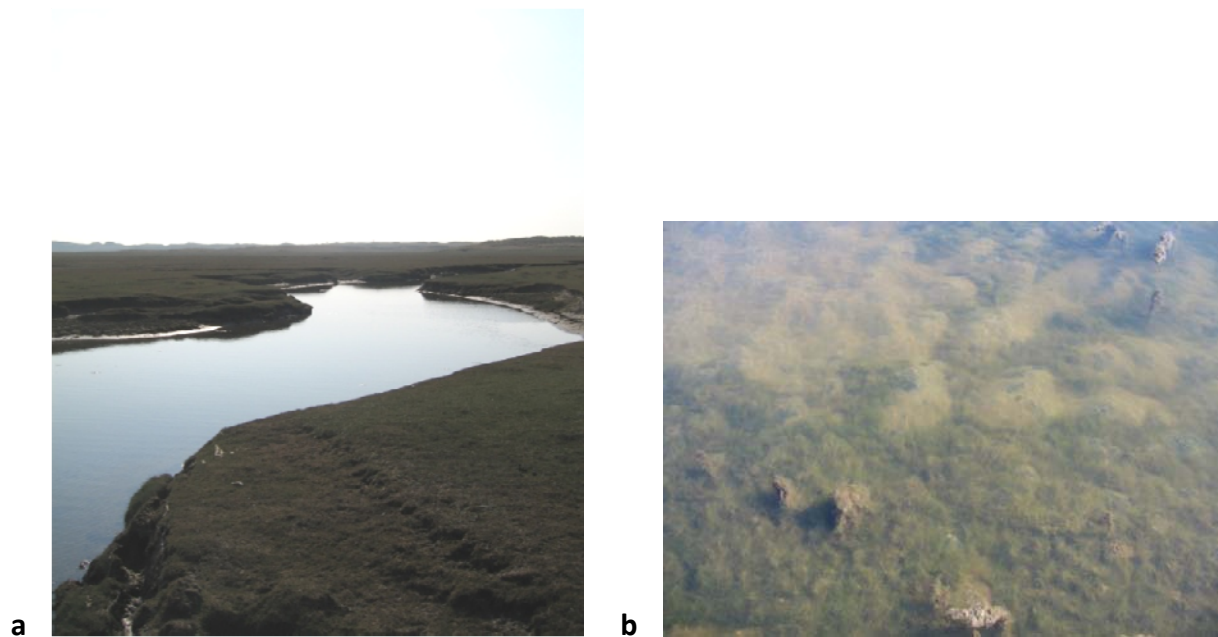


Fig. 5. The tidal creek in the bight of Königshafen, Sylt Germany, sampling site for the marine population; a: overview of the sampling site, b: close-up view.

The freshwater population (UPF) (Fig. 4) was sampled in mid May 2011 in the Pilesø of the “put&take” fishponds in Uge, southern Denmark. This fishpond is a former gravel pit, thus it has no surface afflux or efflux but is groundwater fed. The digging ended in the late 1980s. It has been used for angling since 2000. The fishpond measures approximately 5 ha and the maximal depth is 12 m. Due to the fact that this fishpond originates from gravel dredging, it has a specific conformation with a short shallow shore and then a very steep slope. The sampling was done in the near-shore area with a water depth of up to 1 m, with shrimp and carp nets. The vegetation is a mixture of reed, bushes growing in the shallow water and water plants. The ground is mostly sandy to muddy with small areas of gravel (Fig. 6). Other known fish species in this pond are eel (*Anguilla anguilla*), rainbow trout (*Oncorhynchus mykiss*), roach (*Rutilus rutilus*) and carp (*Cyprinus carpio*). The fishpond is stocked with eel, carp and rainbow trouts every year. These trouts have a minimum weight of 800 g.

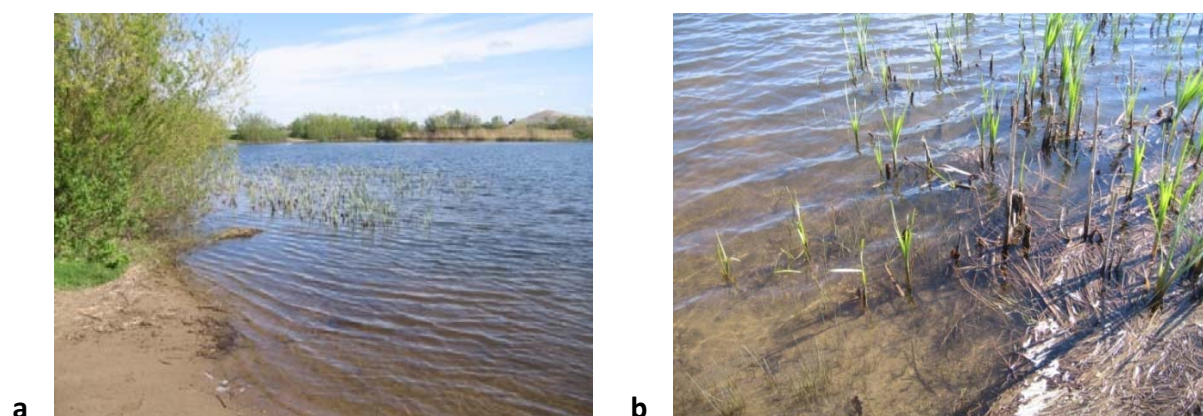


Fig. 6. The Pilesø of the Uge “put&take” fishponds in southern Denmark, sampling site for the freshwater population; a: overview of the sampling site, b: close-up view.

The first anadromous population (HSS) (Fig. 4) was sampled in the end of March 2011 in a ditch draining into the Ho bight, south of Sønderballe. A bow net was placed there over night, which filled out the ditch completely and the migrating fish were trapped and collected in the morning. This ditch is in spring approximately 0.5 m wide and 0.4 m deep. The salinity-measurements confirm that the ditch contains freshwater at the sampling site (Tab. 3). The ground is sandy with hardly any water plants.



Fig. 7. The ditch south of Ho, draining to the Ho bight in the background, southern Denmark; sampling site for an anadromous population; a: overview of the sampling site, b: close-up view.

A second anadromous population (BKD) (Fig. 4) was sampled in Kisbæk, a creek belonging to the Brede Å river system. This river system allows for a barrier-free upstream migration from the Sylt-Rømø bight. This sampling took place in mid April 2011. At this time of the year the creek is about 1.5 m wide and up to 0.6 m deep in the sampling area. The sampling was done with shrimp nets, due to the scarce vegetation in the creek and a very low content of reed on the bank (Fig. 8). The sediment in the creek is mainly gravel with sandy flats in between.



Fig. 8. The sampling site in the Kisbæk, Brede Å system, southern Denmark, a: overview of the sampling site, b: close-up view.

The third anadromous population (RJF) (Fig. 4) from Rømø, Denmark was sampled in the “put&take” fishpond north of Juvre in the beginning of May 2011. The sampling was again done with shrimp nets. This fishpond has a connection to the Wadden Sea, thus the salinity ranges between 13 ‰ and 20 ‰ depending on the tides. This drainage is used for migration by three-spined sticklebacks. The fishpond is about 900 m long and maximally 35 m wide; with a maximal depth of 2.5 m. At the sampling site the pond was up to 1.4 m deep. The shore is very steep with reed on both sides of the fishpond and water plants reaching the surface (Fig. 9). The ground was very muddy with a high content of organic sludge. For angling the pond is stocked with eel (*Anguilla anguilla*), rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*). Further common shrimp (*Crangon crangon*) and common cockle (*Cerastoderme edule*) were found.

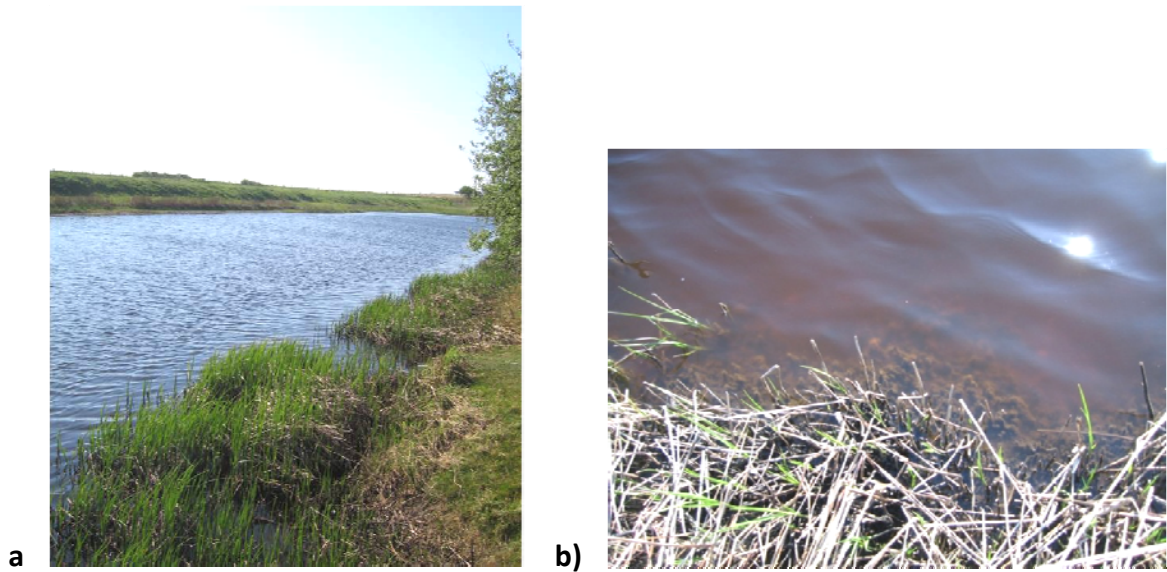


Fig. 9. The “put&take” fishpond north of Juvre, Rømø, Denmark; sampling site for an anadromous population, a: overview of the sampling site, b: close-up view.

3.3 Data acquisition

3.3.1 Photographs

The photographs of the three-spined sticklebacks for the geometric morphometric analyses were taken from the left body side in a standardised procedure. The camera, an Olympus Digitalkamera E-3 with an Olympus Digital 50mm 1:2 Macro ED lens, was placed on a stand 0.5 m above the base plate. To ensure standardised conditions both the base plate and the camera were adjusted horizontally by a water level (“KAISER Action Level Cross digitale Kamera-Wasserwaage”). To avoid shacking or disorientation of the camera a remote-control release was used. The fish were partially embedded in beluga lenses (*Lens culinares*) filled 17 mm high in a box with the right body side downwards. Due to this a plane position for every fish could be reached despite the individual differences in size and body diameter, which is essential for comparable photographs. As far as possible all specimens were similarly orientated while taking the images. Additionally, a scale bar and the individual number were placed in the box (Fig. 10). This box was then placed on the base plate of the stand. The fish were lighted up by four 7 W energy-efficient lamps (TS ELECTRONIC). The setup is shown in Fig. 11.

Tab. 3. List of the sampling sites and their basic data; Abbr. – population abbreviation.

| Site | Abbr. | Water -body type | Width at sampling point [m] | Depth at sampling point [m] | Vegetation | Salinity [‰] | Ground | Other fish species |
|-----------------------------------|-------|---------------------|-----------------------------------|-----------------------------------|--|-----------------|-----------------|----------------------------|
| Brede Å system, Kisbæk | BKD | creek | 1.5 | 0.3 - 0.6 | scarce, reed and other water plants | 0.0 | sand and gravel | |
| Ho bight, south of Sønderballe | HSS | ditch | 0.5 | 0.4 | very scarce | 0.0 | sand | |
| Rømø, Juvre | RJF | fishpond | 30.0 | 0.2 - 1.4 | reed, water plants | 13.2 - 20.0 | putrid mud | eel, trout, carp |
| Sylt, Königshafen | SPK | tidal creek | 0.3 - 4.5 | 0.1 - 1.2 | dwarf eelgrass undefined algae | 27.8 | sand / mud | |
| Uge | UPF | fishpond | 100.0 | 0.1 - 1.0 | reed, near shore bushes, water plants | 1.0 | sand-mud mix | eel, trout, roach, carp |



Fig. 10. Example of a typical photograph used for this study, showing the left body side of the specimen in a plane position with erected dorsal spines and an erected anal spine, including the scale bar and the individual number of each specimen.

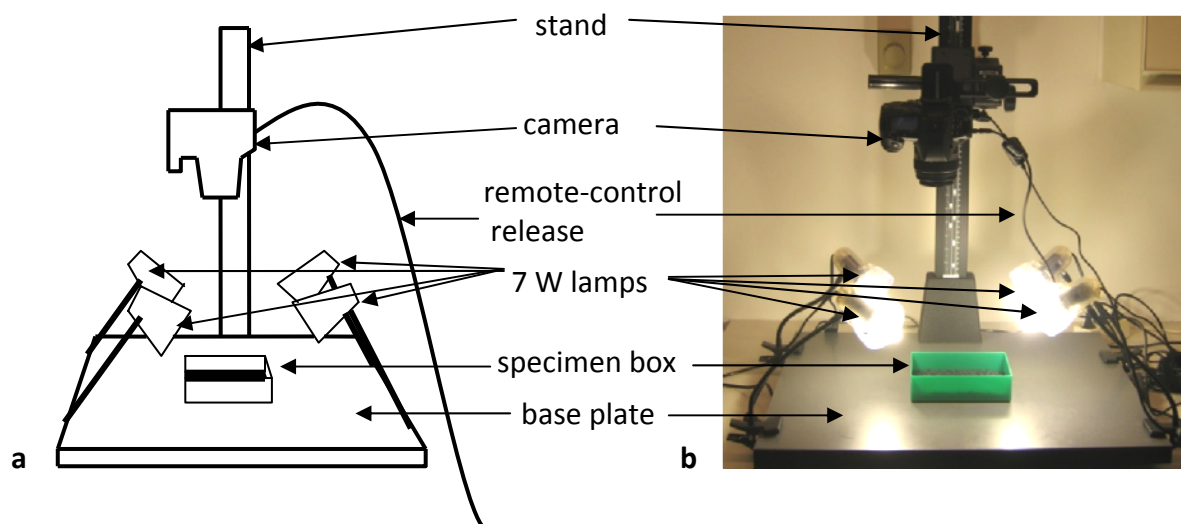


Fig. 11. The set up for the standardised photographs; a: draft, b: actual working place.

3.3.2 Geometric morphometrics

A tps file was built from all the images by tpsUtil (Rohlf 2010c). To capture the overall body shape and some structures (e.g. the fins, the position of the spines, head structures like the eye, the mouth and the lower jaw), which in previous geometric morphometric analyses of three-spined sticklebacks have shown variations (e.g. Walker, 1997; Kitano *et al.*, 2007; Wund *et al.*, 2008; Aguirre and Akinpelu, 2010), 45 landmarks (lms) were chosen. 18 of these lms (lm 28 – lm 45) were defined as sliding landmarks (slms) in tpsUtil (Rohlf 2010c), describing the outline where no anatomically defined lms were possible. The remaining 27 anatomical lms were set on homologous structures on each individual. The lms 1 – 3, 5, 7 – 9, 11, 13 and 15 – 17 have for example been previously used by Walker (1997), the lms 4, 14, 25 and 26 by e.g. Kitano *et al.* (2007), the landmark (lm) 18 by Wund *et al.* (2008) and the lms 19, 20 and 22 have been previously used by e.g. Aguirre and Akinpelu (2010). The number of lms in this study is higher than in most studies on three-spined sticklebacks. This allows a much more efficient and detailed analysis of the shape with the possibility to find small morphological changes and trends in body shape and morphological traits which otherwise may be missed. Further the high number of lms gives the analyses a higher power. The lm digitizing was done in tpsDig2 (Rohlf 2010a). For the exact position and definition of every landmark see Fig. 12 and Tab. 4. The landmark types in Tab. 4 are defined after Bookstein (1991). For a better visualisation of the results, 44 links between lms (Fig. 13 and Tab. 5) were defined to describe the outline of the body, the eye, the mouth, the basis of the pectoral fin and the shape of the ectocoracoid. This was done in tpsUtil (Rohlf 2010c).

To assess changes in particular body regions, several interlandmark distances were computed from the raw coordinates of the digitised lms. For the exact definitions of these interlandmark distances see Tab. 6. All length measurements are calculated relative to the SL; the centroid sizes (cs) of the eye, of the ectocoracoid, and of the caudal peduncle are calculated relative to the cs of the body. The cs of the head is computed relative to the cs of the trunk, and the cs of the eye is additionally calculated relative to the cs of the head.

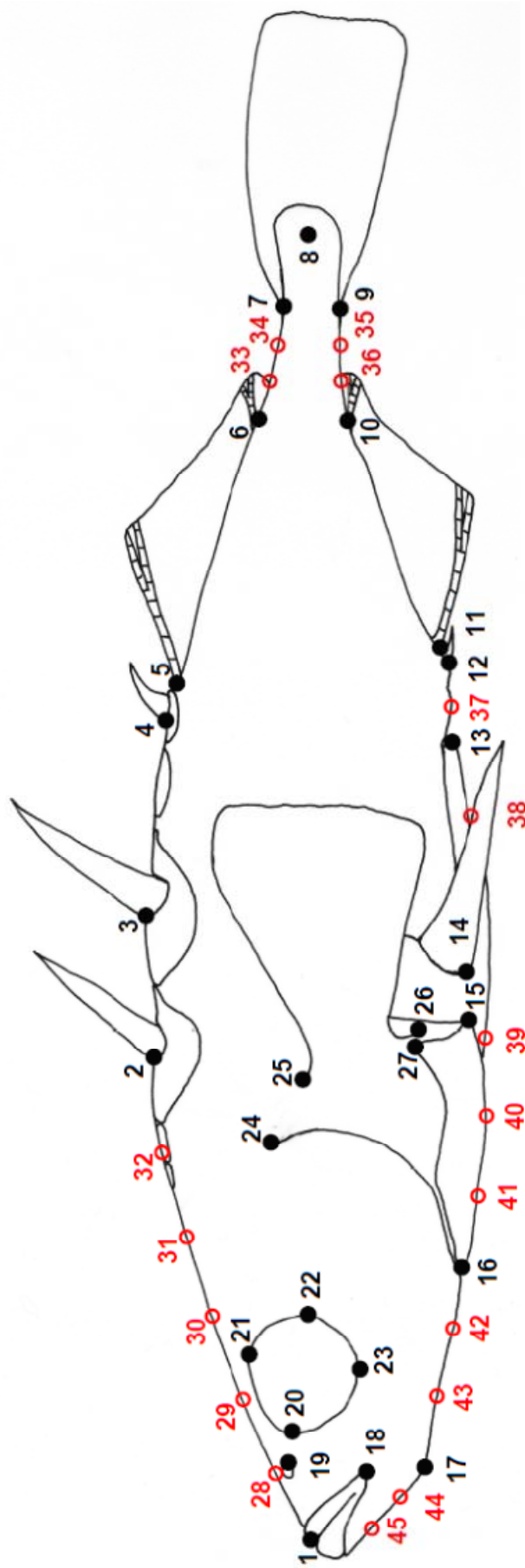


Fig. 12. Schematic illustration of a three-spined stickleback with the locations of the 27 anatomic landmarks 1 – 27 (black, filled circles) and 18 sliding landmarks 28 – 45 (red, open circles) used for the geometric morphometric analyses.

Tab. 4. Definitions of anatomical landmarks and sliding landmarks, additionally the landmark-type is given (defined after Bookstein (1991)); Lm definition – landmark definition, Lm nr – landmark number, Lm type – landmark type, slm – sliding landmark.

| Lm nr. | Lm definition | Lm type |
|---------------|--|----------------|
| 1 | Anterior tip of the upper lip | 3 |
| 2 | Anterior intersection of the first dorsal spine with the pterygiophore III on the dorsal outline | 1 |
| 3 | Anterior intersection of the second dorsal spine with the pterygiophore IV on the dorsal outline | 1 |
| 4 | Anterior intersection of the third dorsal spine with the pterygiophore VI on the dorsal outline | 1 |
| 5 | Anterior base of the first dorsal fin ray | 1 |
| 6 | Posterior base of the last dorsal fin ray | 1 |
| 7 | Dorsal origin of the caudal fin membrane | 1 |
| 8 | Borderline between the last vertebrae and the hypural fan | 1 |
| 9 | Ventral origin of the caudal fin membrane | 1 |
| 10 | Posterior base of the last anal fin ray | 1 |
| 11 | Anterior base of the first anal fin ray | 1 |
| 12 | Anterior intersection of the anal spine with its pterygiophore | 1 |
| 13 | Posterior tip of the ventral process of the pelvic girdle | 2 |
| 14 | Antero-ventral base of the left pelvic spine | 3 |
| 15 | Posterior tip of the ectocoracoid | 2 |
| 16 | Anterior tip of the ectocoracoid | 2 |
| 17 | Posterior tip of the angular | 3 |
| 18 | Posterior point of the mouth | 3 |
| 19 | Posterior point of the nostril | 3 |
| 20 | Most anterior point of the eye | 3 |
| 21 | Most dorsal point of the eye | 3 |
| 22 | Most posterior point of the eye | 3 |
| 23 | Most ventral point of the eye | 3 |
| 24 | Dorsal origin of the operculum membrane | 1 |
| 25 | Dorsal origin of the pectoral fin | 1 |
| 26 | Ventral origin of the pectoral fin | 1 |
| 27 | Dorsal tip of the ectocoracoid | 2 |
| 28 - 32 | Dorsal outline of the head | slm |
| 33 - 34 | Dorsal outline of the caudal peduncle | slm |
| 35 - 36 | Ventral outline of the caudal peduncle | slm |
| 37 | Ventral outline of the abdomen | slm |
| 38 | Ventral outline of the ventral process of the pelvic girdle | slm |
| 39 - 41 | Ventral outline of the breast | slm |
| 42 - 43 | Ventral outline of the throat | slm |
| 44 - 45 | Ventral outline of the chin | slm |

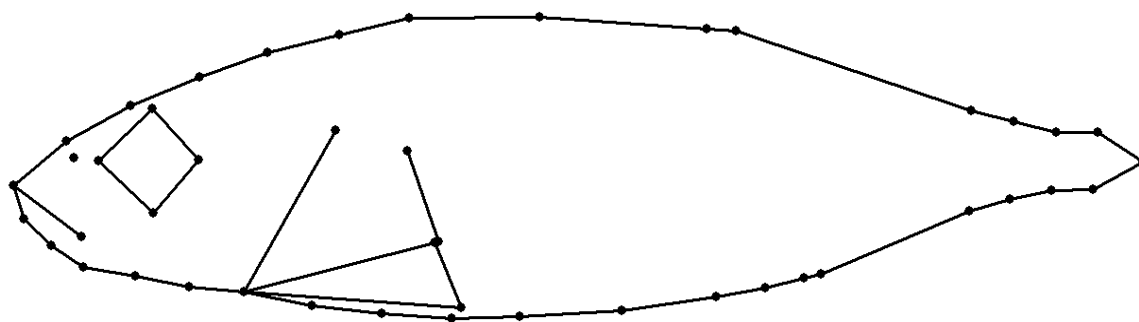


Fig. 13. The 44 links between the landmarks for a better visualisation of the results show: the outline, the mouth, the eye, the ectocoracoid, the basis of the pectoral fin and a line between the dorsal origin of the operculum membrane and the anterior tip of the ectocoracoid.

Tab. 5. List of the links between the landmarks for a better visualisation of the results.

| Link describing | Included landmarks in the connected order |
|---------------------------|--|
| Outline | 1-28-29-30-31-32-2-3-4-5-6-33-34-7-8-9-35-36-10-11-12-37-13-38-14-39-40-41-16-42-43-17-44-45 |
| Mouth | 1-18 |
| Eye | 20-21-22-23 |
| Ectocoracoid | 15-16-27 |
| Basis of the pectoral fin | 25-26 |
| Operculum-Ectocoracoid | 24-16 |

Tab. 6. Alphabetic list of all length and centroid size measurements done on the landmark configurations; cs – centroid size, rel – relative, SL – standard length.

| Abbreviation | Definition | Included landmarks |
|-----------------------|--|---|
| Body cs | Cs of the entire lm configuration | 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45 |
| Head cs | Cs of the head | 1, 16, 17, 18, 19, 20, 21, 22, 23, 24, 28, 29, 30, 31, 32, 42, 43, 44, 45 |
| rel anal fin | Length of the basis of the anal fin (from the first to the last anal fin ray) relative to the SL. | 10, 11 |
| rel body height | Body height (between the anterior intersection of the first dorsal spine with the pterygiophore III on the dorsal outline and the posterior tip of the ectocoracoid) relative to the SL. | 2, 15 |
| rel caud.ped. cs | Cs of the caudal peduncle relative to the cs of the body. | 6, 7, 8, 9, 10 |
| rel caud.ped. dorsal | Dorsal length of the caudal peduncle (between the last dorsal fin ray and the dorsal origin of the caudal fin membrane) relative to the SL. | 6, 7 |
| rel caud.ped. height1 | Anterior height of the caudal peduncle (between the last dorsal fin ray and the last anal fin ray) relative to the SL. | 6, 10 |

| Abbreviation | Definition | Included landmarks |
|--------------------------|--|--|
| rel caud.ped. height2 | Posterior height of the caudal peduncle (between the dorsal and the ventral origin of the caudal fin membrane) relative to the SL. | 7, 9 |
| rel caud.ped. ventral | Ventral length of the caudal peduncle (between the last anal fin ray and the ventral origin of the caudal fin membrane) relative to the SL. | 9, 10 |
| rel dorsal fin | Length of the basis of the dorsal fin (from the first to the last dorsal fin ray) relative to the SL. | 5, 6 |
| rel ds1-ds2 | Distance between the anterior intersection of the first dorsal spine with the pterygiophore III on the dorsal outline and the anterior intersection of the second dorsal spine with the pterygiophore IV on the dorsal outline relative to the SL. | 2, 3 |
| rel ds2-ds3 | Distance between the anterior intersection of the second dorsal spine with the pterygiophore IV on the dorsal outline and the anterior intersection of the third dorsal spine with the pterygiophore VI on the dorsal outline relative to the SL. | 3, 4 |
| rel ds1-ds3 | Distance between the anterior intersection of the first dorsal spine with the pterygiophore III on the dorsal outline and the anterior intersection of the third dorsal spine with the pterygiophore VI on the dorsal outline relative to the SL. | 2, 4 |
| rel ectocoracoid cs | Cs of the ectocoracoid relative to the cs of the body. | 15, 16, 27 |
| rel eye cs | Cs of the eye relative to the cs of the head. | 20, 21, 22, 23 |
| rel eye cs2 | Cs of the eye relative to the cs of the body. | 20, 21, 22, 23 |
| rel head cs | Cs of the head relative to the cs of the trunk. | 1, 16, 17, 18, 19, 20, 21, 22, 23, 24, 28, 29, 30, 31, 32, 42, 43, 44, 45 |
| rel head length | Length of the head (from the anterior tip of the upper lip to the dorsal origin of the operculum membrane) relative to the SL. | 1, 24 |
| rel jaw | Length of the angular (from the anterior tip of the upper lip to the posterior point of the angular) relative to the SL. | 1, 17 |
| rel mouth | Length of the mouth (from the anterior tip of the upper lip to the posterior point of the mouth) relative to the SL. | 1, 18 |
| rel pectoral fin | Length of the basis of the pectoral fin (from the dorsal to the ventral origin of the pectoral fin) relative to the SL. | 25, 26 |
| rel snout | Length of the snout (from the anterior tip of the upper lip to the anterior point of the eye) relative to the SL. | 1, 20 |
| SL | Standard length (from the anterior tip of the upper lip to the hypural fan). | 1, 8 |
| Trunk cs | Cs of the trunk | 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 24, 25, 26, 27, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41 |

3.4 Data analyses

3.4.1 Geometric morphometrics

Geometric morphometric analyses are based on shape coordinates. The shape of an object is unaffected by its size, position, and orientation. The raw coordinates achieved by the digitalisation contain information about the shape and the size of the object along with further information about position and orientation. Shape coordinates are usually estimated by a Procrustes superimposition. For this the centroid and the centroid size (cs) of every object has to be computed. The centroid is the arithmetic mean of the landmark configuration, the cs is then calculated by the square root of the summed squared distances of the landmarks and their centroid (Bookstein, 1991). The Procrustes superimposition involves three steps and results in centered, scaled, and rotated landmarks, so-called Procrustes shape coordinates. The first step is the translation of all landmark configurations to the same centroid (usually the origin of the coordinate system) to standardise for position. The second step is the scaling of all landmark configurations to the same centroid size. Finally the landmark configurations are rotated until the sum of the squared Euclidian distances between the homologous landmarks is minimal, see Fig. 14 (Mitteroecker and Gunz, 2009). With these Procrustes shape coordinates the actual analyses are proceeded.

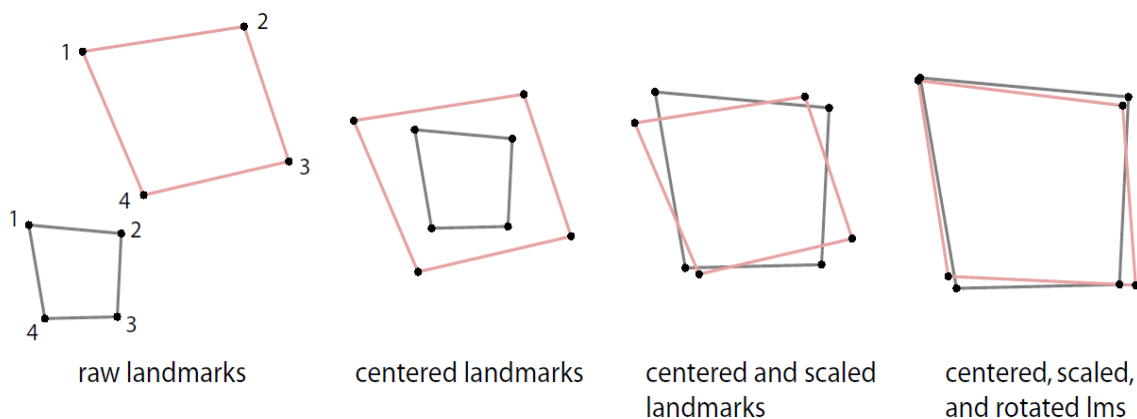


Fig. 14. The three steps of Procrustes superimposition: translation to a common origin, scaling to unit centroid size and rotation to minimize the sum of squared Euclidian distances among the homologous landmarks. The resulting landmark coordinates are called Procrustes shape coordinates (Mitteroecker and Gunz, 2009).

For a better visualisation of the results, several deformation grids based on the thin-plate spline (TPS) method of Bookstein (1989) were calculated. “The TPS interpolation function from a template configuration to a target configuration is usually applied to the vertices of a regular grid so that the shape differences between the two geometries can be read from the deformation of this grid [(Fig. 15)]. When the actual shape differences are subtle, the deformation can be extrapolated by an arbitrary factor to ease the interpretation of the grid.” Mitteroecker and Gunz (2009).

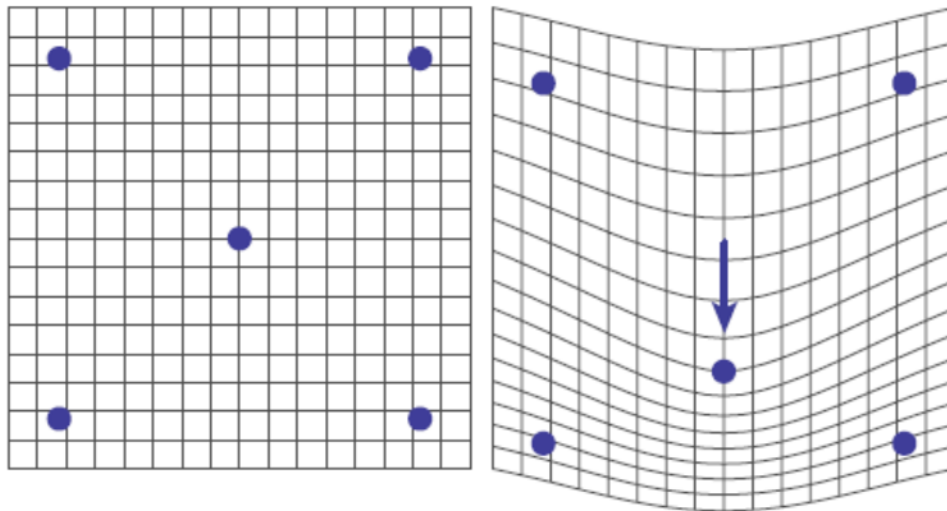


Fig. 15. A template configuration (left) and a target configuration (right) of five landmarks each. The deformation grid on the right illustrates the thin-plate spline function between these configurations as applied to the left regular grid—it is a visualization of the differences between the two shapes (Mitteroecker and Gunz, 2009).

3.4.2 Applied geometric morphometric analyses and statistics

The Procrustes superimposition and sliding of the sliding lms was done in tpsRelw (Rohlf 2010b). These slid Procrustes shape coordinates were then exported to Microsoft Excel and prepared for the import in Wolfram Mathematica (version 8 and version 8.4) where all further statistical and geometric morphometric analyses were done. For all the geometric morphometric analyses and the permutation tests in Wolfram Mathematica two scripts (MorphometricsFunction5.11 and MorphometricsFunction5-13) by Mitteroecker P. and Gunz P. (unpublished) were applied.

The Relative Warp Analyses were calculated and plotted. Relative Warps are an equivalent to a Principal Component Analysis (PCA) (Valentin *et al.*, 2008). Therefore only the PCA is explained in the following. The first Principal Component (PC) is the direction of the highest variance in the data set. The second PC axis is orthogonal to the first one and in the direction of the second greatest variance. All further axes are orthogonal to all the previous axes and in order of decreasing variance. The data points were projected orthogonal on the new axes. The so achieved PC scores are uncorrelated with each other. Each point in a PCA plot corresponds to one lm configuration. The original second Principal Component showed mainly upward and downward arching effects and hardly any biological changes in the body shape. According to Valentin *et al.* (2008) the reason for these arching effects are slight differences in posture when the images are taken and the lms captured and not biological or preservation effects. Therefore the PC 2 was projected out of the data space to correct for this error source. And the analyses presented in the results were done with the resulting data set.

To compare the different populations and ecotypes with each other and to examine the sexual dimorphism different methods were used. From the Procrustes shape coordinates population means were calculated and plotted similar to a PCA. Further a between-group PCA was computed, which is a PCA of the group means and the individuals are projected into the space of these Principal Components (Mitteroecker and Bookstein, 2011). For a better visualisation of the results, deformation grids were computed to show the effects on body shape.

The Procrustes superimposition scales all individuals to the same size, but allometric effects still remain (Rohlf and Marcus, 1993). All specimens analysed in this study were adult (SL > 32 mm). According to Walker (1993) in (Walker, 1997) the body shape allometry is small after reaching sexual maturity and should therefore not affect the results of this study. Nevertheless to test for allometric effects a shape regression was computed.

Several pair wise permutation tests were conducted to compare group means of the different ecotypes, populations, and sexes. The *p*-values were corrected by the Bonferroni correction (multiplied by 95). The significance level was determined at *p* = 0.05.

4. Results

4.1 General patterns

Fig. 16 shows the consensus configuration of all examined specimens.

The PCA of the group means of the sexes of each population calculated from the Procrustes shape coordinates of all examined specimens (Fig. 17) shows a clustering of males and females. This indicates a strong sexual dimorphism along the first axis, which explains 87% of the total shape variance. The males of all five populations cluster on the negative end of the first axis, while the females cluster on the positive end. The second axis explains 5.14% of the variance and separates the different populations from each other. For the male individuals, the marine population (SPK) is the most negative one on this axis, the freshwater population (UPF) has the most positive value and the three anadromous populations are in between, closer to each other than to the marine or the freshwater population. The distribution of the females is different. The marine and the freshwater population have similar values on the first axis but are separated by the second axis, the freshwater population again on the more positive end. The three anadromous populations (BKD, HSS, RJF) are better separated from the other females by the first axis instead of the second, but also these cluster together. The grids in Fig. 17 reflect the body shape deformations along the first two PC axes. The deformation grids for the PC1 are extrapolated by a factor of 0.04, they reflect the male and female specific body shape. The grids for the PC2 are extrapolated by a factor of 0.03. The remaining PCs, explaining about 8 % of the variance in body shape, were uncorrelated to sex or ecotype (results not presented).

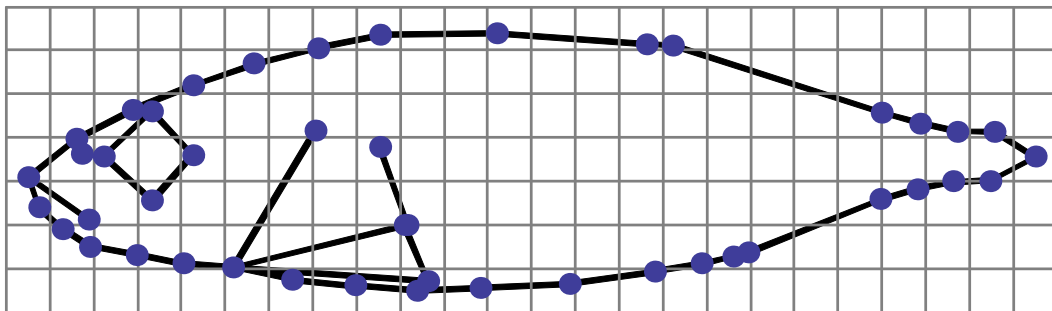


Fig. 16. Consensus configuration of all examined specimens.

The between-group PCA (Fig. 18) shows the scores for each specimen, giving better insights into the variability of the body shape. The first axis separates the sexes, the males with the negative values and the females with the positive values. The ecotypes are partially separated by the second axis, but some clusters have large overlapping areas. A permutation test with 5000 permutation shows a significant group mean difference between the males of the marine and the freshwater ecotype ($p = 0.019$), see chapter 4.3. The males of these two ecotypes hardly overlap; meaning that the males of these two ecotypes can be separated by the used set of lms. In contrast to this, the cluster of the marine ecotype completely overlaps with the anadromous ecotype cluster. These two ecotypes cannot be separated properly by this method. The females of the three ecotypes overlap with each other; their classification with between-group PCAs of this lm configuration is not possible.

4.2 Sexual dimorphism

For the analysis of sexual dimorphism all females of all five populations were grouped and all males of all five populations were grouped regardless of which ecotype they belong to. Fig. 19 shows the mean body shape of all examined females and males. Slight differences are visible, especially in the head size, the caudal peduncle and the ectocoracoid, but for a better visualisation the deformation grids in Fig. 20 were calculated.

The deformation grids show the mean shape differences between females (Fig. 20a) and males (Fig. 20b) (pooled over all five populations) extrapolated by a factor of two. The grids indicate that the females have smaller heads, hence also smaller head structures like the eye, the snout, the mouth and the lower jaw. The abdominal region is clearly enlarged in females, similar to the larger abdomen the distance between the second and the third dorsal spine is longer, and the anal fin is shorter. The caudal peduncle is narrower and slightly longer in females. The basis of the pectoral fin appears longer in males and the shape of the ectocoracoid is different. The first dorsal spine is in a more anterior position in males than it is in females.

From the 19 interlandmark distances computed from the landmark configuration, 13 show significant differences after Bonferroni correction between the two sexes pooled over all 5 populations. In the following only the significant results are mentioned, for the results of all measurements and two-group permutation tests see Tab. 7.

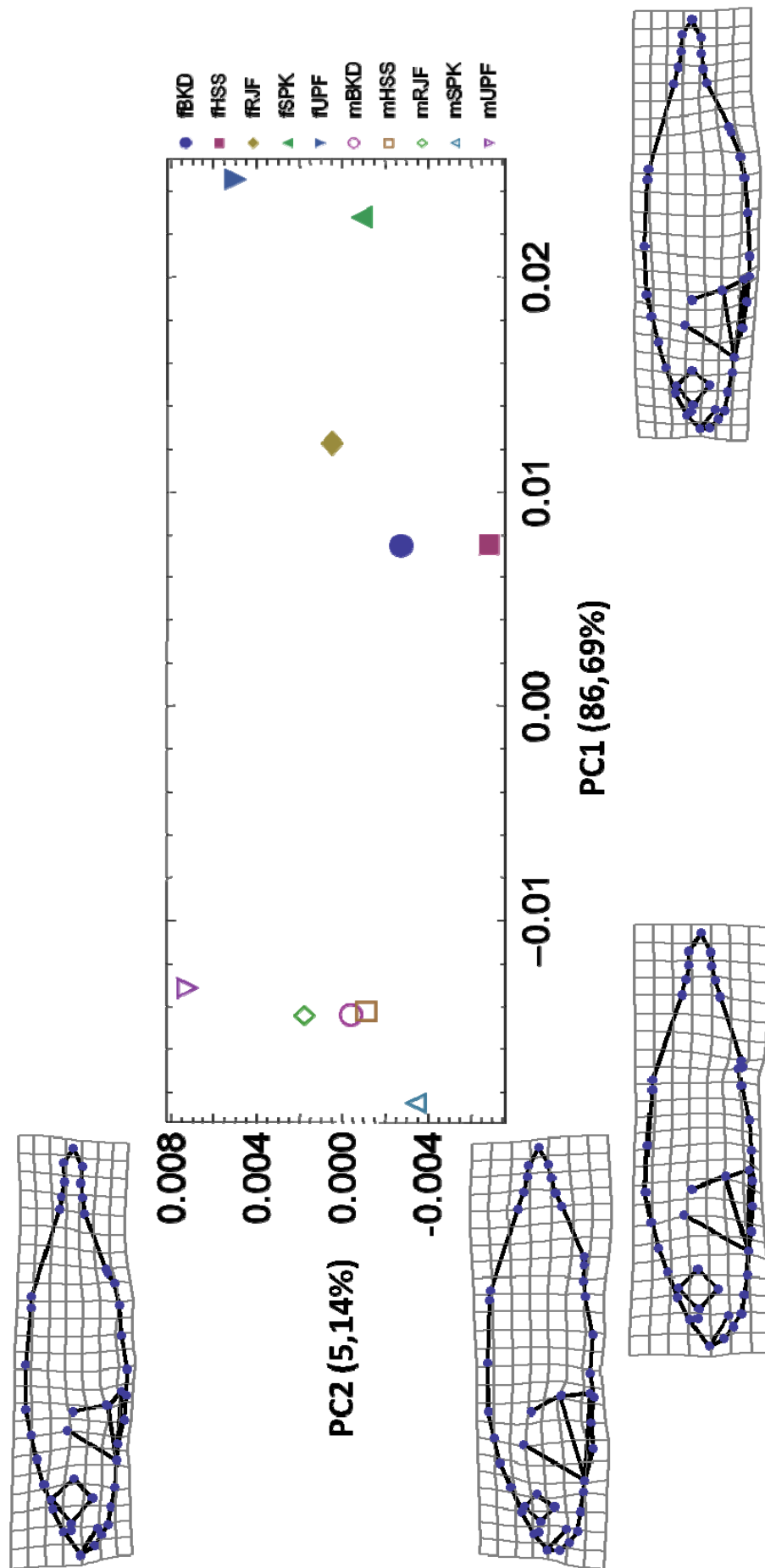


Fig. 17. The first and second axes of a Principal Component analysis of the group means of the Procrustes shape coordinates of all males and females of each population, and the deformation grids representing the changes in body shape along the axes. The deformation grids along the first axis are extrapolated by a factor of 0.04 and the grids of the second axis by a factor of 0.03. m – male, f – female, for population abbreviations see Tab. 2.

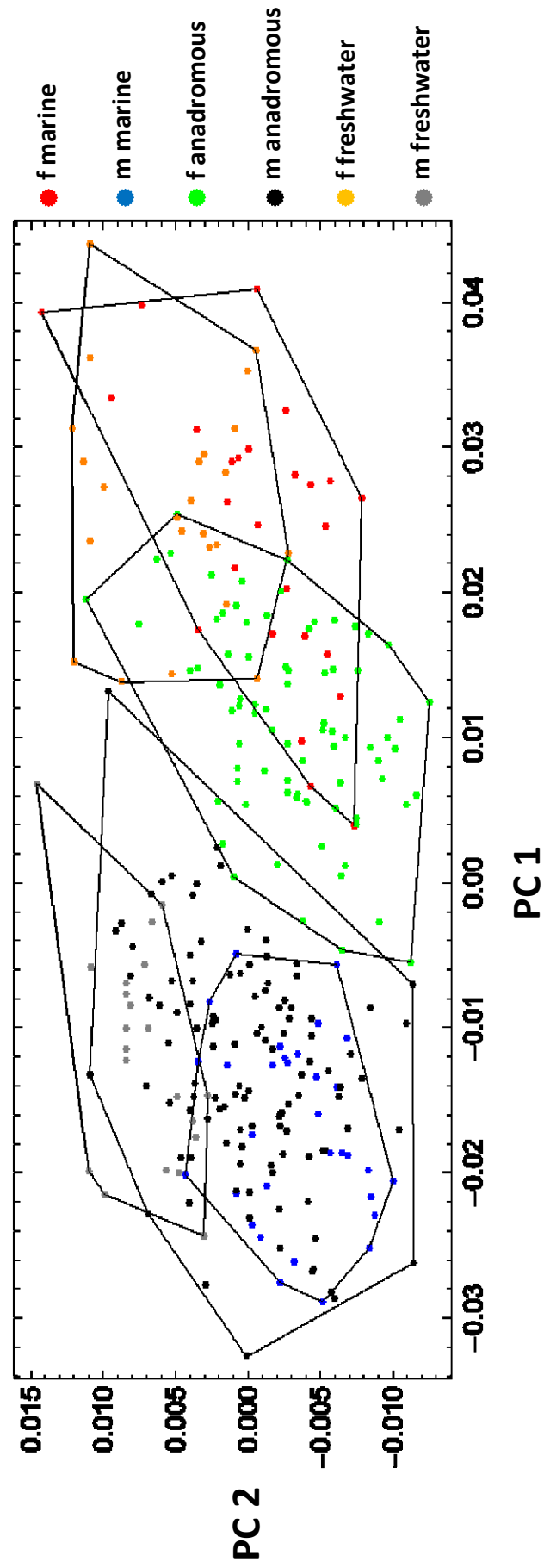


Fig. 18. The first and second axes of the between group Principal Component Analysis of all examined specimens, the lines show the convex hulls including all female or male specimens of one ecotype. m – male, f – female.

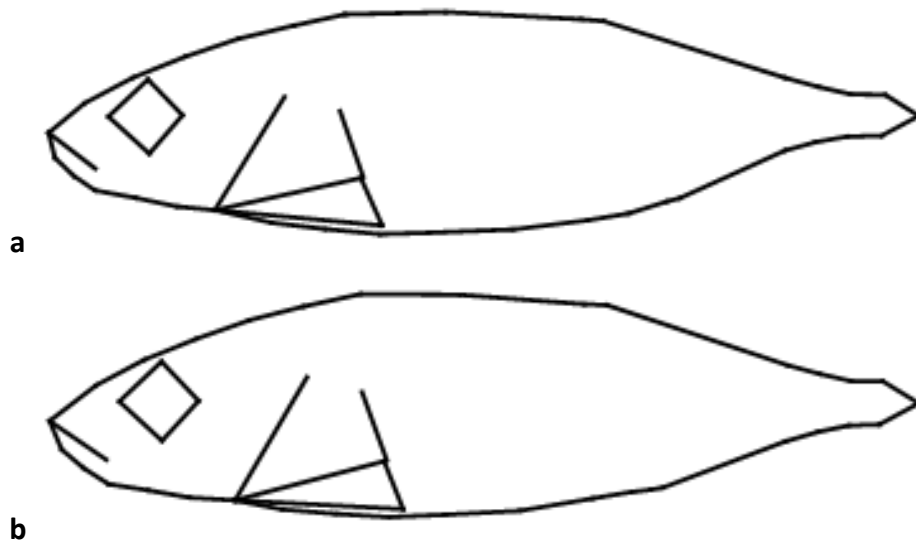


Fig. 19. Body shape of the mean female (a) and mean male (b) pooled over all 5 populations.

As indicated by the grids, the relative cs of the head ($p = 0.019$) and the relative head length ($p = 0.019$) are significantly larger in males. The mouth ($p = 0.019$), the snout ($p = 0.019$) and the lower jaw ($p = 0.019$) are also significantly longer in males. There is no significant difference in the cs of the eye relative to the cs of the head, but there is a significant difference when it is calculated relative to the cs of the body ($p = 0.019$). Another significant difference is found in the relative cs of the ectocoracoid ($p = 0.019$), which is larger in males. To quantify the changes in the angle of the basis of the pectoral fin, the angle in the posterior tip of the ectocoracoid was calculated. This angle is significantly larger in males ($p = 0.019$). The eye-catching differences between the two sexes in the caudal peduncle were only partially supported by the measures. The dorsal length of the caudal peduncle (between lm 6 and lm 7) is significantly larger in females ($p = 0.019$) while the ventral length (between lm 9 and lm 10) is not significantly different. Similar to this, the anterior height of the caudal peduncle is not significantly different ($p = 0.057$) but the posterior height of the caudal peduncle is larger in males ($p = 0.019$). The sexual dimorphism in the abdominal region can for example be seen in the significantly longer distance between the second and the third dorsal spine in females ($p = 0.019$), hence also the distance between the first and the third dorsal spine is longer in females ($p = 0.019$). More subtle, even on the extrapolated grids, is the significantly larger body height in males ($p = 0.019$). The sexual dimorphism is also reflected by the two-group permutation tests with 5000 permutations of the entire lm configuration of the two sexes within each population, see Tab. 8.

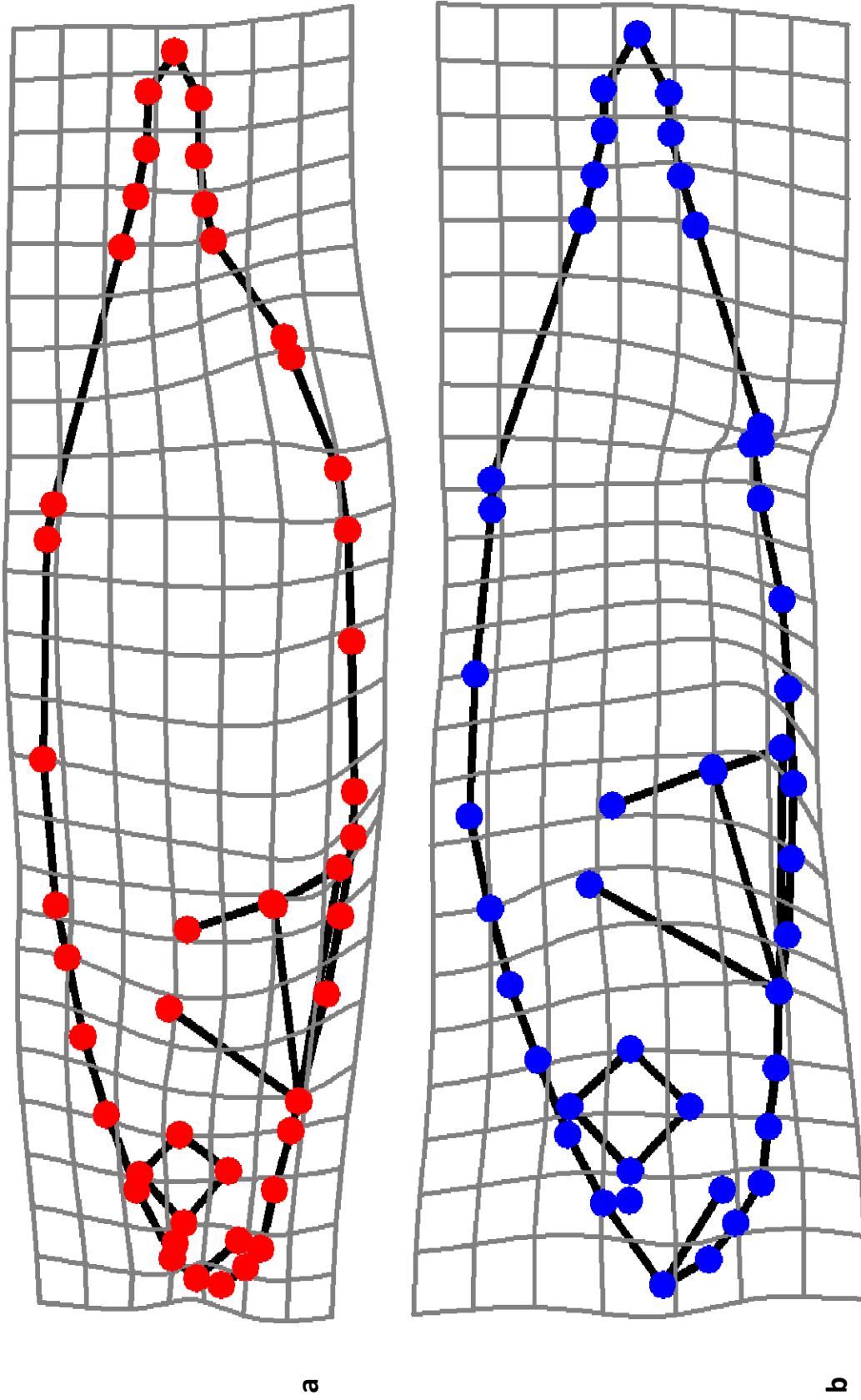


Fig. 20. Deformation grids showing the mean shape differences between females (a) and males (b) including all 5 populations. The mean female was used as the template grid and the mean male as the target grid; the result was extrapolated by a factor of 2.

Tab. 7. List of all minimum, mean, and maximum values of the length measurements and centroid size measurements of all females (f) and males (m) pooled over all five populations. Additionally the standard deviations and the *p*-values as well as the Bonferroni corrected *p*-values of the two-group permutation tests with 5000 permutations are given; for the abbreviations see Tab. 6, corr.*p*-v. – Bonferroni corrected *p*-value, max – maximum, min – minimum, n.s. – not significant, *p*-value – original *p*-value, sd – standard deviation, * - marking significant measurements after Bonferroni correction.

| Structure abbreviation | sex | min | mean | max | sd | <i>p</i> -value | corr. <i>p</i> -v. |
|------------------------|-----|--------|--------|--------|-------|-----------------|--------------------|
| rel head cs | f | 0.282 | 0.309 | 0.359 | 0.014 | 0.0002 | 0.019* |
| | m | 0.296 | 0.336 | 0.369 | 0.012 | | |
| rel eye cs | f | 0.175 | 0.190 | 0.216 | 0.008 | 0.053 | n.s. |
| | m | 0.175 | 0.192 | 0.216 | 0.008 | | |
| rel eye cs2 | f | 0.035 | 0.039 | 0.044 | 0.002 | 0.0002 | 0.019* |
| | m | 0.038 | 0.042 | 0.047 | 0.002 | | |
| rel head length | f | 0.253 | 0.276 | 0.310 | 0.010 | 0.0002 | 0.019* |
| | m | 0.266 | 0.299 | 0.325 | 0.010 | | |
| rel snout | f | 0.060 | 0.072 | 0.090 | 0.006 | 0.0002 | 0.019* |
| | m | 0.067 | 0.083 | 0.098 | 0.006 | | |
| rel mouth | f | 0.059 | 0.069 | 0.083 | 0.005 | 0.0002 | 0.019* |
| | m | 0.065 | 0.079 | 0.088 | 0.005 | | |
| rel jaw | f | 0.076 | 0.086 | 0.109 | 0.006 | 0.0002 | 0.019* |
| | m | 0.077 | 0.099 | 0.117 | 0.006 | | |
| rel pectoral fin | f | 0.072 | 0.080 | 0.095 | 0.004 | 0.0002 | 0.019* |
| | m | 0.073 | 0.084 | 0.097 | 0.005 | | |
| rel ectocoracoid cs | f | 0.059 | 0.070 | 0.080 | 0.004 | 0.0002 | 0.019* |
| | m | 0.059 | 0.072 | 0.083 | 0.004 | | |
| ecto-angle | f | 38.914 | 60.399 | 74.998 | 7.463 | 0.0002 | 0.019* |
| | m | 49.167 | 65.068 | 78.327 | 6.299 | | |
| rel dorsal fin | f | 0.177 | 0.218 | 0.247 | 0.013 | 0.431 | n.s. |
| | m | 0.169 | 0.220 | 0.255 | 0.016 | | |
| rel anal fin | f | 0.094 | 0.133 | 0.179 | 0.015 | 0.0002 | 0.019* |
| | m | 0.120 | 0.150 | 0.192 | 0.015 | | |
| rel caud.ped.dorsal | f | 0.075 | 0.117 | 0.149 | 0.013 | 0.0002 | 0.019* |
| | m | 0.075 | 0.111 | 0.176 | 0.015 | | |
| rel caud.ped.ventral | f | 0.081 | 0.113 | 0.159 | 0.013 | 0.012 | n.s. |
| | m | 0.078 | 0.109 | 0.150 | 0.014 | | |
| rel caud.ped.height1 | f | 0.070 | 0.085 | 0.105 | 0.009 | 0.001 | n.s. |
| | m | 0.066 | 0.089 | 0.128 | 0.009 | | |
| rel caud.ped.height2 | f | 0.038 | 0.048 | 0.054 | 0.003 | 0.0002 | 0.019* |
| | m | 0.043 | 0.051 | 0.057 | 0.003 | | |
| rel caud.ped.cs | f | 0.058 | 0.073 | 0.092 | 0.006 | 0.861 | n.s. |
| | m | 0.059 | 0.073 | 0.090 | 0.006 | | |

| Structure abbreviation | sex | min | mean | max | sd | <i>p</i> -value | corr. <i>p</i> -v. |
|------------------------|-----|-------|-------|-------|-------|-----------------|--------------------|
| rel ds1-ds2 | f | 0.090 | 0.116 | 0.140 | 0.008 | 0.118 | n.s. |
| | m | 0.095 | 0.115 | 0.138 | 0.008 | | |
| rel ds2-ds3 | f | 0.128 | 0.154 | 0.189 | 0.011 | 0.0002 | 0.019* |
| | m | 0.120 | 0.144 | 0.167 | 0.010 | | |
| rel ds1-ds3 | f | 0.238 | 0.270 | 0.299 | 0.012 | 0.0002 | 0.019* |
| | m | 0.229 | 0.259 | 0.288 | 0.012 | | |
| rel body height | f | 0.222 | 0.247 | 0.271 | 0.010 | 0.0002 | 0.019* |
| | m | 0.224 | 0.253 | 0.288 | 0.010 | | |

Tab. 8. The *p*-values and the Bonferroni corrected *p*-values (Bonf.corr. *p*-value) of the two-group permutation tests with 5000 permutations, comparing the group means of males and females of each population, * - marking significant results after Bonferroni correction, for population abbreviations see Tab. 2.

| Population | <i>p</i> -value | Bonf.corr. <i>p</i> -value |
|------------|-----------------|----------------------------|
| BKD | 0.0002 | 0.019* |
| HSS | 0.0002 | 0.019* |
| RJF | 0.0002 | 0.019* |
| SPK | 0.0002 | 0.019* |
| UPF | 0.0002 | 0.019* |

4.3 Ecotype differentiation

Fig. 17 shows that the sexual dimorphism is larger than the ecotype differences. For body shape variations between the different ecotypes only the males were investigated as explained in chapter 3.1. For the males of all five populations a group mean of the total *lm* configuration was calculated and a PCA was computed with the resulting data (Fig. 21). The PC1 separates the three ecotypes, the marine at the positive end, the freshwater on the negative end, and in between cluster the three anadromous populations. This axis explains approximately 50% of the variance in this data set. The second axis, explaining approximately 22%, separates especially the three anadromous populations from each other. The island population from Rømø (RJF) is on the most negative end of PC2, while the marine (SPK) and the freshwater (UPF) ecotype are on the most positive end. From the anadromous populations the HSS is closest to the SPK and UPF population. The grids in Fig. 21 are extrapolated by a factor of 0.03. The grids for PC1 reflect the body shape differences between the freshwater and the marine ecotype. Eye-catching are the more streamlined body shape, the larger eyes and the ectocoracoid is smaller in this grid at the negative end of

the PC1. The specific ecotype differences are stated below. The grids for PC2 show at the positive end a mixture of the freshwater and the marine ecotype, and at the negative end it reflects the body shape of the RJF population. The three anadromous populations seem to differ from each other especially in the size of the abdominal region and the length of the ventral side of the ectocoracoid. These two parameters are smaller in the RJF population than in the HSS population. Furthermore, the eye is smaller and the caudal peduncle narrower in the RJF population. By contrast, the length of the mouth and the throat are enlarged in the RJF population. The remaining PCs, explaining about 28 % of the variance in body shape, were unrelated to the ecotypes (results not presented).

For the following analyses the three anadromous populations are pooled into one anadromous ecotype.

Fig. 22 shows the actual mean male body shape of each ecotype. The marine ecotype is deeper bodied, with the largest ectocoracoid. There is a gradual change from the marine to the anadromous and further to the freshwater ecotype. The freshwater ecotype is more streamlined with a narrower caudal peduncle, a smaller ectocoracoid and a different angle of the pectoral fin. In contrast to the sexual dimorphism, no considerable changes in the head structures are visible. A pairwise permutation test with 5000 permutations between the different ecotypes gives only a significant group mean difference after the Bonferroni correction between the marine and the freshwater ecotype ($p = 0.019$). The group means of the marine and the anadromous ecotype as well as the group means of the anadromous and the freshwater ecotype are not significantly different after the Bonferroni correction with this high correction factor (Tab. 9).

Tab. 9. The p -values and the Bonferroni corrected p -values (Bonf.corr. p -value) of the two-group permutation tests with 5000 permutations, comparing the group means of the males of the three ecotypes; n.s. – not significant, * - marking significant results after Bonferroni correction.

| Ecotypes | p -value | Bonf.corr. p -value |
|---------------------------|------------|-----------------------|
| Marine vs. anadromous | 0.001 | n.s. |
| Anadromous vs. freshwater | 0.013 | n.s. |
| Marine vs. freshwater | 0.0002 | 0.019* |

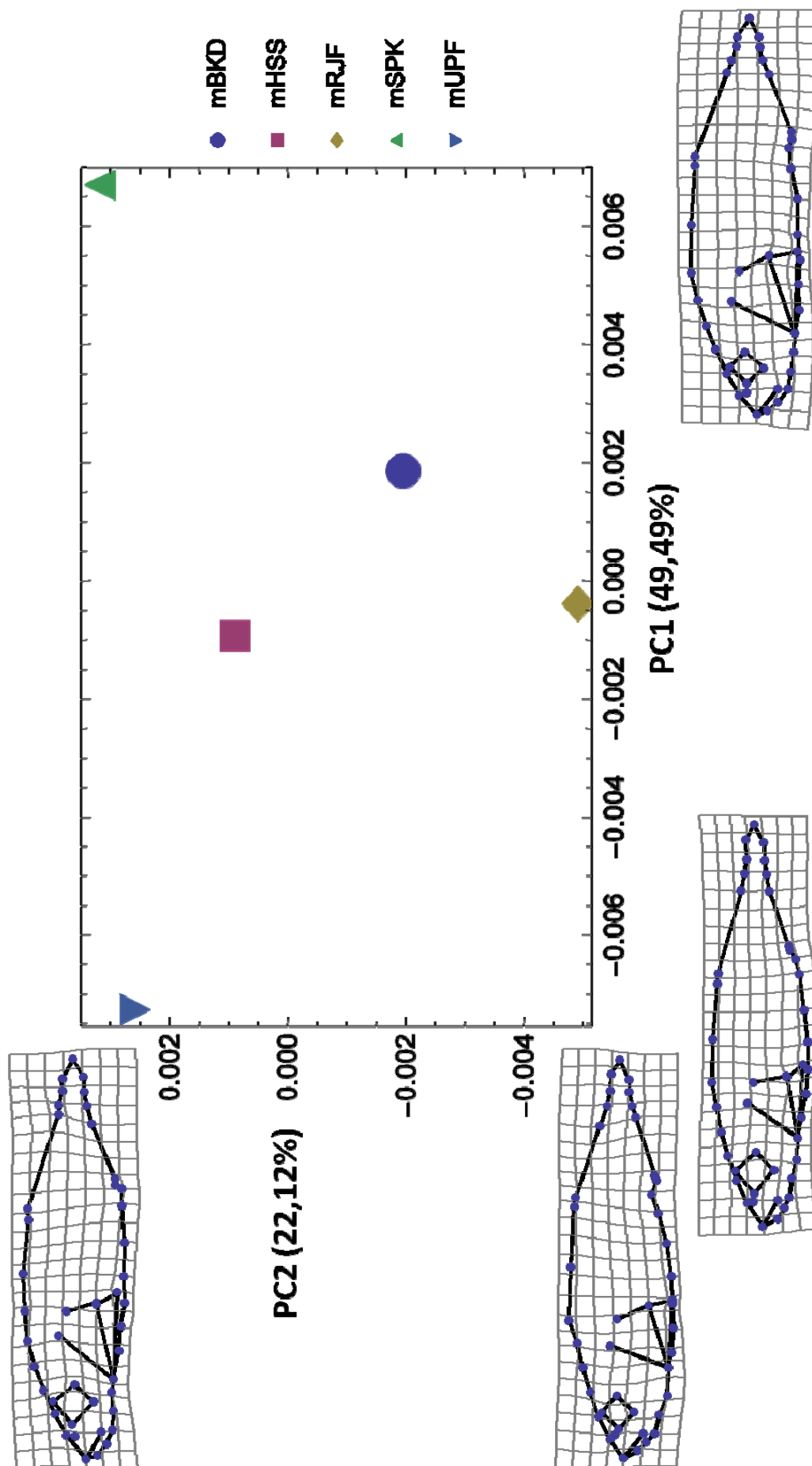


Fig. 21. The first and second axes of the Principal Component Analysis of the group means of the Procrustes shape coordinates of all male specimens of each population and the deformation grids representing the changes in body shape along the axes. The deformation grids along both axes are extrapolated by a factor of 0.03; green triangle – marine, blue inverted triangle – freshwater, other symbols anadromous, for population abbreviations see Tab. 2.

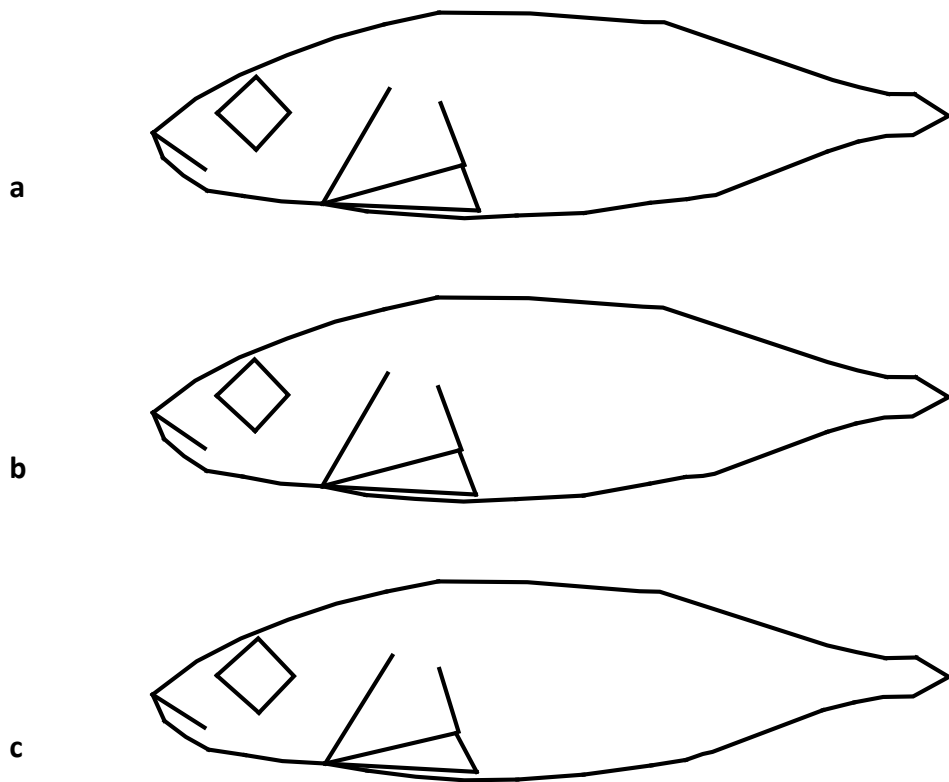


Fig. 22. The actual body shape of the mean male of the marine (a), the anadromous (b) and the freshwater (c) ecotype.

For a better visualisation of the mean changes in body shape deformation grids of the different ecotypes were computed and extrapolated by a factor of 3. Note especially the shift in the deformation grids of the anadromous population towards the freshwater ecotype body shape when compared with the marine ecotype and towards the marine ecotype body shape when compared to the freshwater ecotype.

Fig. 23 shows the resulting deformation grids of the marine ecotype warped against the anadromous ecotype. The marine ecotype is deeper bodied, with a short and high caudal peduncle. The dorsal fin looks longer in the marine three-spined sticklebacks deformation grid, but the result is not significant after Bonferroni-correction. Changes are also observable in the size and shape of the ectocoracoid and hence the angle of the pectoral fin. In the head structures like the eye, the jaw, the mouth, or the snout length, no differences occur. Neither in the abdominal region nor in the distance between the dorsal spines morphological changes can be observed, even at this extrapolation factor. Although these grids show some trends in body shape differentiation, no significant differences are found after the Bonferroni correction of the measurement results (Tab. 10).

When warping the mean male anadromous three-spined stickleback against the mean male freshwater three-spined stickleback more morphological changes in several structures are found (Fig. 24). The eye of the freshwater ecotype is significantly larger whether it is calculated relative to the cs of the head ($p = 0.019$) or the cs of the entire body ($p = 0.019$). The length of the head looks larger in the freshwater ecotype, as does the snout length but both measures do not show significant results in the two-group permutation tests. Also the length of the mouth and the lower jaw are similar in both ecotypes. Large changes are found in the shape and the cs of the ectocoracoid. Both the cs of the ectocoracoid ($p = 0.019$) and the angle at the posterior tip of the ectocoracoid ($p = 0.019$) are significantly larger in the anadromous ecotype. This leads to changes in the position of the ventral origin of the pectoral fin. The freshwater ecotype has a significantly smaller body height ($p = 0.019$) and a longer and narrower caudal peduncle, this leads to a more streamlined body shape. The length of the caudal peduncle measured on the ventral side is significantly longer in the freshwater ecotype ($p = 0.019$), measured on the dorsal side no significant difference in the length of the caudal peduncle is found. Also in the height of the caudal peduncle no significant difference is found. Neither in the length of the basis of the dorsal fin nor in the length of the basis of the anal fin could any difference be examined. For all measuring results and computed permutation tests see Tab. 10.

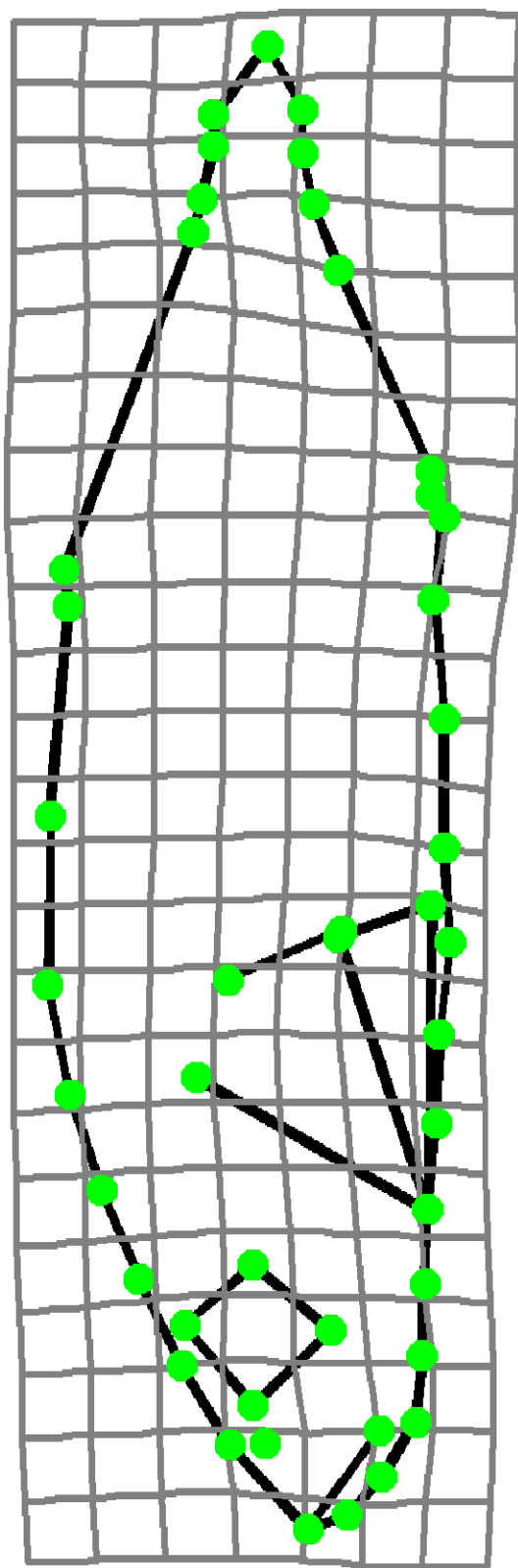
Fig. 25 shows the mean differences in body shape between the two ecotypes (marine and freshwater) from the opposite ends of PC1 of the group mean PCA (Fig. 21). Here similar changes in the different structures occur like described above at the anadromous ecotype warped against the freshwater ecotype, but they are more eye-catching. The marine ecotype is significantly deeper bodied ($p = 0.038$), with a short and high caudal peduncle. The elongation of the caudal peduncle in the freshwater ecotype can only be seen as a trend since the group means do not differ significantly after the Bonferroni correction. Neither the length of the bases of the dorsal nor of the anal fin show significant differences between these two ecotypes. The deformation grids of these two ecotypes warped against each other indicate similarities in the position of the dorsal spines and also in the size of the pelvic girdle. In contrast to this there are huge differences in the pectoral girdle. Both the cs of the ectocoracoid ($p = 0.019$) and the angle at the posterior tip of the ectocoracoid ($p = 0.019$) are significantly larger in the marine ecotype. This leads to a change in the position of the ventral

origin of the pectoral fin. In the marine ecotype the ventral origin of the pectoral fin is clearly posterior to the dorsal origin. In the freshwater ecotype the ventral origin of the pectoral fin is shifted into a more anterior position, resulting in an almost vertical position. The cs of the head and the length of the head are not differing between these two ecotypes. Further the grids indicate no differences in the length of the mouth, lower jaw and snout. But the freshwater ecotype has a clearly enlarged eye (calculated relative to the cs of the head, $p = 0.019$). For the measured values and the complete list of significance levels see Tab. 10.

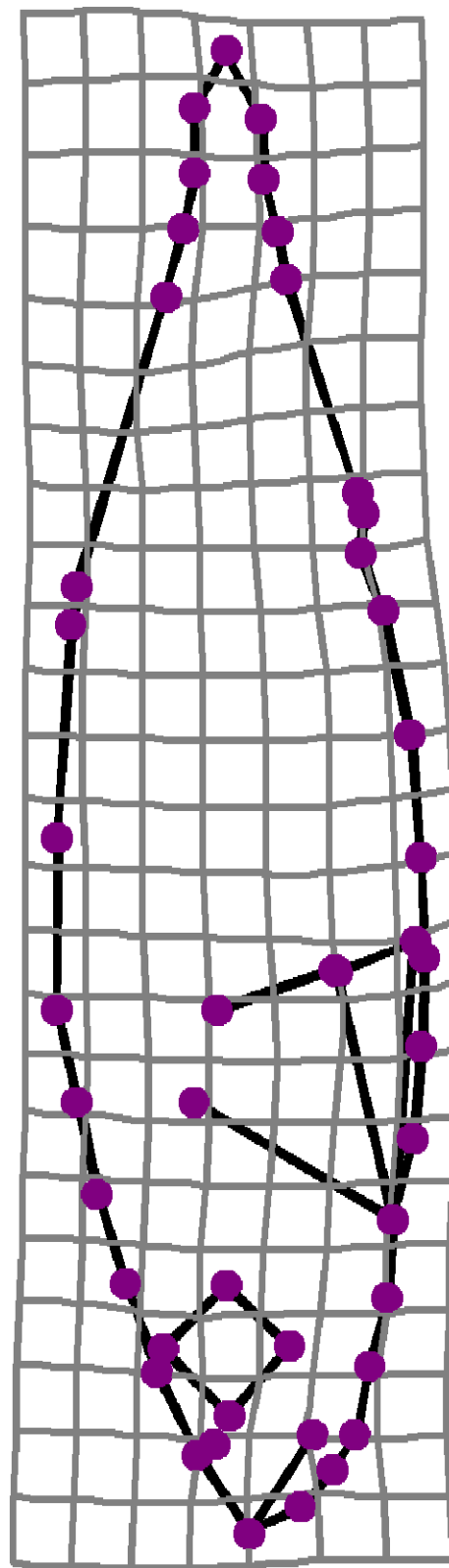
4.4 Allometric effects

Although all examined specimens are within a SL size range of 41.20 – 58.91 mm allometric effects on the body shape occur. The effect of size on body shape was estimated via a shape regression and showed that 5.48% of shape variance are explained by allometric effects. In Fig. 26 these effects are visualized. The grids reflect the body shape 10 standard deviations from the mean lm configuration in the positive and the negative direction. Fig. 26a shows the hypothetical shape of a very small individual. Here the large head and head structures like the mouth, the snout and the eye are conspicuous. Further the ectocoracoid is smaller, but the basis of the pectoral fin is longer. The angle at the posterior tip of the ectocoracoid is hardly changing. The relative distance between the dorsal spines is smaller, the same holds true for the abdominal region. By contrast, the anal fin is relatively longer in these smaller specimens. The opposite direction of the allometric effects leads to a smaller head in relation to the trunk, hence all head structures are smaller. These very large specimens have further a larger ectocoracoid, abdomen and the distance between the second and third dorsal spine is longer. The basis of the pectoral fin and the anal fin is shorter. The caudal peduncle does not show any differences.

Since allometry may confound differences between adult individuals, it was thus regressed out of the data set and all the analyses were redone (results not presented). Since there were no qualitative differences in the results the data including the allometric effect were used for this study. The direction of the allometric effect is very similar to the one observed for the sexual dimorphism because the females were on average larger than the males (chapter 4.2). Especially in this case where the allometric effect is linked to specific biological changes the regression could lead to a loss of information.



a



b

Fig. 23. Deformation grids showing the mean shape differences between the males of the marine ecotype (a) and the males of the anadromous ecotype (b), the marine male was used as template grid and the anadromous male was used as target grid, the result was extrapolated by a factor of 3.

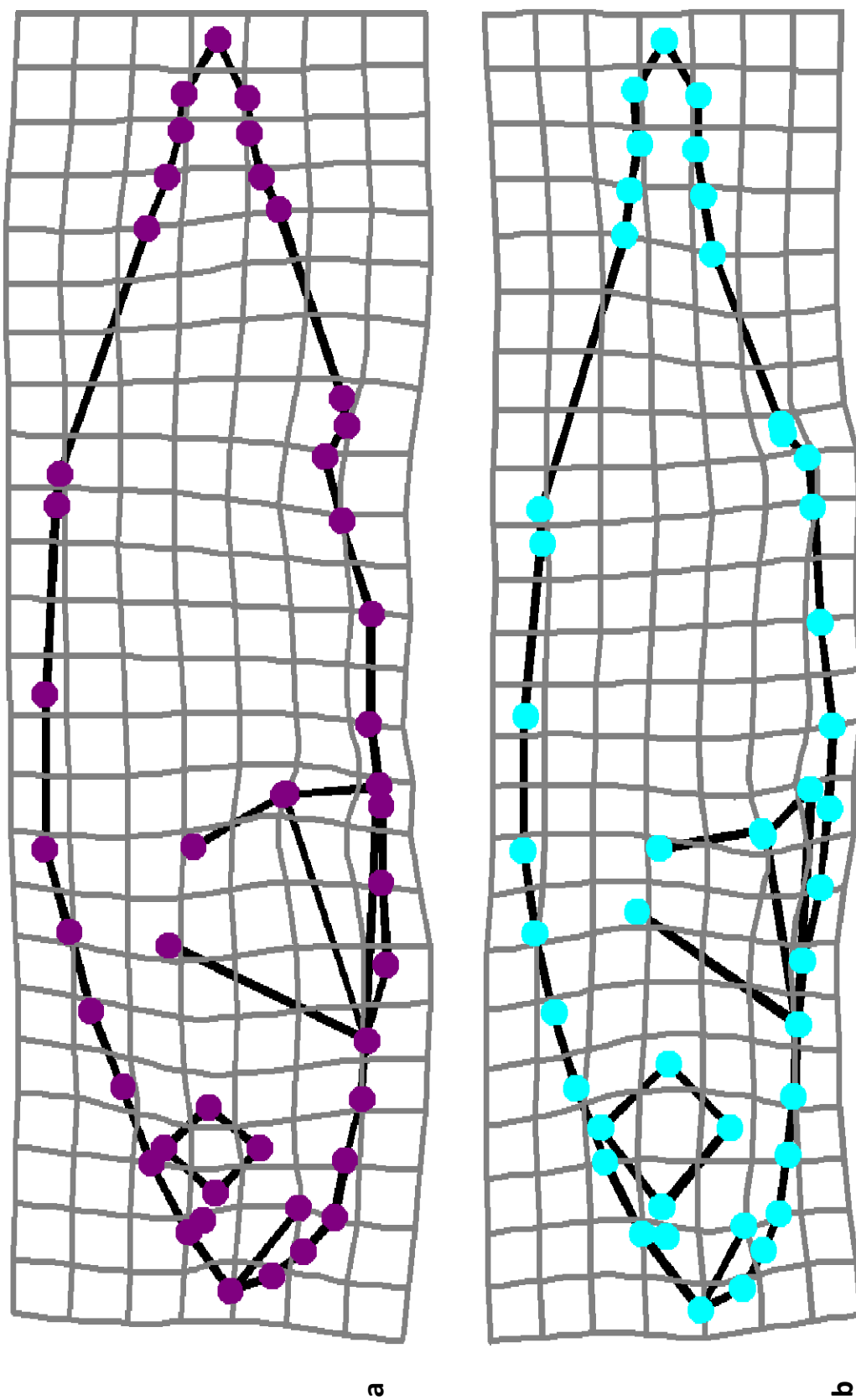


Fig. 24. Deformation grids showing the mean shape differences between the males of the anadromous ecotype (a) and the males of the freshwater ecotype (b), the anadromous male was used as template grid and the freshwater male was used as target grid, the result was extrapolated by a factor of 3.

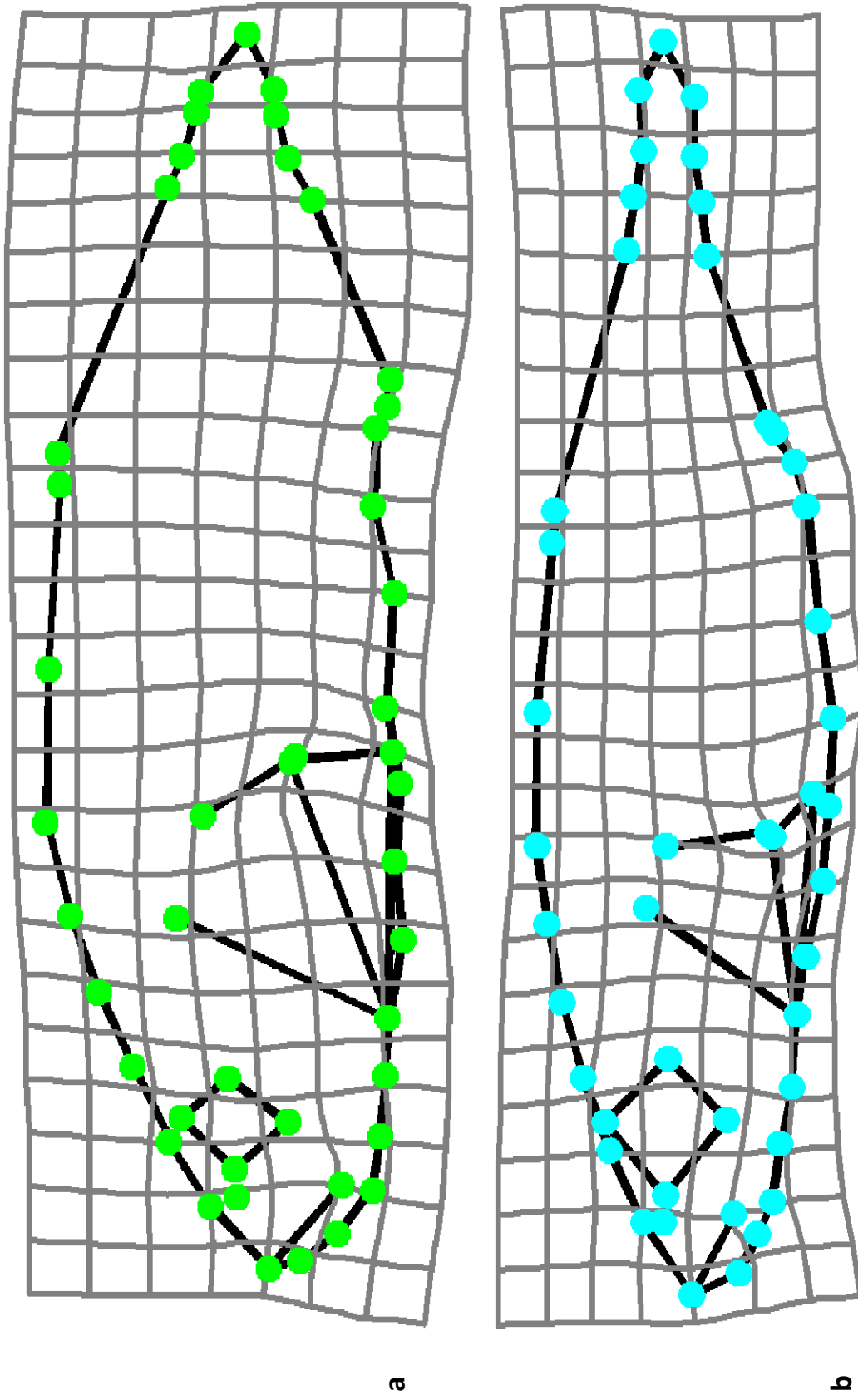


Fig. 25. Deformation grids showing the mean shape differences between the males of the marine ecotype (a) and the males of the freshwater ecotype (b), the marine male was used as template grid and the freshwater male was used as target grid, the result was extrapolated by a factor of 3.

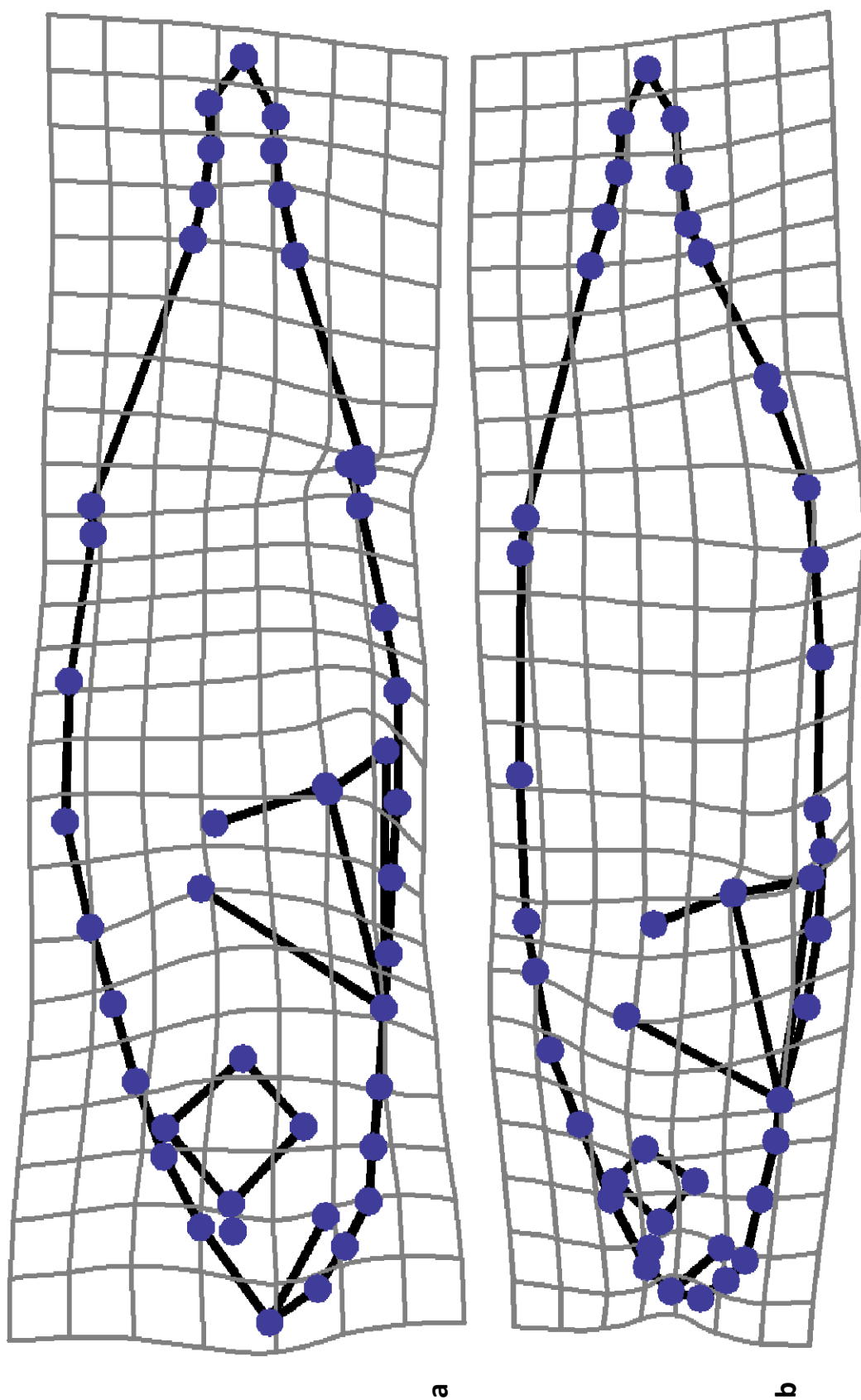


Fig. 26. Deformation grids showing the allometric changes in body shape ten standard deviations in the negative (a) and ten standard deviations in the positive (b) direction.

Tab. 10. List of the minimum, mean and maximum values of the length and centroid size measurements of all males of the three ecotypes, additionally the standard deviations and the *p*-values and Bonferroni corrected *p*-values of the computed two-group permutation tests with 5000 permutations are given; for the abbreviations see Tab. 6, ana – anadromous ecotype, corr.*p*-v. a-fw – Bonferroni corrected *p*-value tested the anadromous against the freshwater ecotype, corr.*p*-v. m-a – Bonferroni corrected *p*-value tested the anadromous against the marine ecotype, corr.*p*-v. m-fw – Bonferroni corrected *p*-value tested the marine against the freshwater ecotype, freshw – freshwater ecotype, marine – marine ecotype, max – maximum, min – minimum, n.s. – not significant, *p*-value a-fw – original *p*-value tested the anadromous against the freshwater ecotype, *p*-value m-a – original *p*-value tested the marine against the anadromous ecotype, *p*-value m-fw – original *p*-value tested the marine against the freshwater ecotype, sd – standard deviation, * – marking significant *p*-values after Bonferroni correction.

| Structure abbreviation | ecotype | min | mean | max | sd | <i>p</i> -value m-a | <i>p</i> -value a-fw | <i>p</i> -value m-fw | corr. <i>p</i> -v. m-a | corr. <i>p</i> -v. a-fw | corr. <i>p</i> -v. m-fw |
|------------------------|---------|-------|-------|-------|-------|---------------------|----------------------|----------------------|------------------------|-------------------------|-------------------------|
| rel head cs | marine | 0.305 | 0.338 | 0.361 | 0.012 | 0.306 | | | n.s. | | |
| | ana | 0.296 | 0.336 | 0.369 | 0.013 | | 0.313 | | | n.s. | |
| | freshw | 0.314 | 0.333 | 0.349 | 0.010 | | | 0.075 | | | n.s. |
| rel eye cs | marine | 0.184 | 0.192 | 0.200 | 0.005 | 0.182 | | | n.s. | | |
| | ana | 0.175 | 0.190 | 0.216 | 0.008 | | 0.0002 | | | 0.019* | |
| | freshw | 0.192 | 0.202 | 0.214 | 0.006 | | | 0.0002 | | | 0.019* |
| rel eye cs2 | marine | 0.039 | 0.042 | 0.045 | 0.001 | 0.052 | | | n.s. | | |
| | ana | 0.038 | 0.042 | 0.047 | 0.002 | | 0.0002 | | | 0.019* | |
| | freshw | 0.041 | 0.044 | 0.047 | 0.002 | | | 0.001 | | | n.s. |

| Structure abbreviation | ecotype | min | mean | max | sd | p-value m-a | p-value a-fw | p-value m-fw | corr. p-v. m-a | corr. p-v. a-fw | corr. p-v. m-fw |
|---------------------------|---------|-------|-------|-------|-------|----------------|-----------------|-----------------|-------------------|--------------------|--------------------|
| rel head length | marine | 0.283 | 0.300 | 0.318 | 0.008 | 0.559 | | | n.s. | | |
| | ana | 0.266 | 0.299 | 0.325 | 0.011 | | 0.843 | | | n.s. | |
| | freshw | 0.282 | 0.299 | 0.311 | 0.008 | | | 0.444 | | | n.s. |
| rel snout | marine | 0.071 | 0.084 | 0.092 | 0.005 | 0.476 | | | n.s. | | |
| | ana | 0.067 | 0.083 | 0.098 | 0.007 | | 0.944 | | | n.s. | |
| | freshw | 0.076 | 0.083 | 0.089 | 0.004 | | | 0.454 | | | n.s. |
| rel mouth | marine | 0.073 | 0.079 | 0.088 | 0.004 | 0.765 | | | n.s. | | |
| | ana | 0.065 | 0.079 | 0.088 | 0.005 | | 0.147 | | | n.s. | |
| | freshw | 0.069 | 0.077 | 0.082 | 0.003 | | | 0.064 | | | n.s. |
| rel jaw | marine | 0.088 | 0.099 | 0.110 | 0.005 | 0.922 | | | n.s. | | |
| | ana | 0.077 | 0.099 | 0.117 | 0.006 | | 0.096 | | | n.s. | |
| | freshw | 0.085 | 0.097 | 0.103 | 0.004 | | | 0.102 | | | n.s. |
| rel pectoral fin | marine | 0.075 | 0.084 | 0.097 | 0.005 | 0.649 | | | n.s. | | |
| | ana | 0.073 | 0.084 | 0.093 | 0.005 | | 0.110 | | | n.s. | |
| | freshw | 0.073 | 0.082 | 0.088 | 0.004 | | | 0.289 | | | n.s. |

| Structure abbreviation | ecotype | min | mean | max | sd | p-value m-a | p-value a-fw | p-value m-fw | corr. p-v. m-a | corr. p-v. a-fw | corr. p-v. m-fw |
|---------------------------|---------|--------|--------|--------|-------|----------------|-----------------|-----------------|-------------------|--------------------|--------------------|
| rel ectocoracoid cs | marine | 0.067 | 0.073 | 0.077 | 0.002 | 0.384 | | | n.s. | | |
| | ana | 0.059 | 0.072 | 0.083 | 0.004 | | 0.0002 | | | 0.019* | |
| | freshw | 0.062 | 0.068 | 0.072 | 0.003 | | | 0.0002 | | | 0.019* |
| ecto-angle | marine | 54.930 | 66.373 | 78.194 | 4.999 | 0.987 | | | n.s. | | |
| | ana | 52.791 | 66.356 | 78.327 | 5.590 | | 0.0002 | | | 0.019* | |
| | freshw | 49.167 | 56.213 | 64.139 | 4.339 | | | 0.0002 | | | 0.019* |
| rel dorsal fin | marine | 0.196 | 0.225 | 0.248 | 0.014 | 0.029 | | | n.s. | | |
| | ana | 0.169 | 0.218 | 0.255 | 0.017 | | 0.724 | | | n.s. | |
| | freshw | 0.211 | 0.227 | 0.250 | 0.009 | | | 0.119 | | | n.s. |
| rel anal fin | marine | 0.120 | 0.149 | 0.190 | 0.017 | 0.442 | | | n.s. | | |
| | ana | 0.120 | 0.152 | 0.192 | 0.015 | | 0.008 | | | n.s. | |
| | freshw | 0.124 | 0.143 | 0.164 | 0.011 | | | 0.123 | | | n.s. |
| rel caud.ped.dorsal | marine | 0.075 | 0.106 | 0.134 | 0.014 | 0.070 | | | n.s. | | |
| | ana | 0.082 | 0.112 | 0.176 | 0.016 | | 0.568 | | | n.s. | |
| | freshw | 0.091 | 0.114 | 0.137 | 0.011 | | | 0.034 | | | n.s. |

| Structure abbreviation | ecotype | min | mean | max | sd | p-value m-a | p-value a-fw | p-value m-fw | corr. p-v. m-a | corr. p-v. a-fw | corr. p-v. m-fw |
|---------------------------|---------|-------|-------|-------|-------|----------------|-----------------|-----------------|-------------------|--------------------|--------------------|
| rel caud.ped.ventral | marine | 0.083 | 0.111 | 0.145 | 0.016 | 0.226 | | | n.s. | | |
| | ana | 0.078 | 0.107 | 0.150 | 0.013 | | 0.0002 | | | 0.019* | |
| | freshw | 0.100 | 0.119 | 0.136 | 0.008 | | | 0.027 | | | n.s. |
| rel caud.ped.height1 | marine | 0.071 | 0.091 | 0.104 | 0.008 | 0.287 | | | n.s. | | |
| | ana | 0.066 | 0.089 | 0.128 | 0.009 | | 0.004 | | | n.s. | |
| | freshw | 0.072 | 0.083 | 0.094 | 0.006 | | | 0.001 | | | n.s. |
| rel caud.ped.height2 | marine | 0.045 | 0.052 | 0.057 | 0.003 | 0.010 | | | n.s. | | |
| | ana | 0.043 | 0.050 | 0.056 | 0.003 | | 0.175 | | | n.s. | |
| | freshw | 0.044 | 0.049 | 0.056 | 0.003 | | | 0.002 | | | n.s. |
| rel caud.ped.cs | marine | 0.061 | 0.072 | 0.085 | 0.006 | 0.977 | | | n.s. | | |
| | ana | 0.059 | 0.072 | 0.091 | 0.006 | | 0.038 | | | n.s. | |
| | freshw | 0.069 | 0.075 | 0.084 | 0.004 | | | 0.059 | | | n.s. |
| rel ds1-ds2 | marine | 0.102 | 0.115 | 0.126 | 0.006 | 0.752 | | | n.s. | | |
| | ana | 0.095 | 0.115 | 0.138 | 0.008 | | 0.726 | | | n.s. | |
| | freshw | 0.103 | 0.114 | 0.126 | 0.006 | | | 0.523 | | | n.s. |

| Structure abbreviation | ecotype | min | mean | max | sd | p-value m-a | p-value a-fw | p-value m-fw | corr. p-v. m-a | corr. p-v. a-fw | corr. p-v. m-fw |
|---------------------------|---------|-------|-------|-------|-------|----------------|-----------------|-----------------|-------------------|--------------------|--------------------|
| rel ds2-ds3 | marine | 0.120 | 0.145 | 0.160 | 0.009 | 0.668 | | | n.s. | | |
| | ana | 0.124 | 0.144 | 0.167 | 0.010 | | 0.394 | | | n.s. | |
| | freshw | 0.131 | 0.146 | 0.161 | 0.007 | | | 0.648 | | | n.s. |
| rel ds1-ds3 | marine | 0.237 | 0.260 | 0.279 | 0.012 | 0.595 | | | n.s. | | |
| | ana | 0.229 | 0.258 | 0.288 | 0.013 | | 0.700 | | | n.s. | |
| | freshw | 0.244 | 0.260 | 0.278 | 0.010 | | | 0.961 | | | n.s. |
| rel body height | marine | 0.238 | 0.254 | 0.269 | 0.008 | 0.635 | | | n.s. | | |
| | ana | 0.227 | 0.255 | 0.288 | 0.010 | | 0.0002 | | | 0.019* | |
| | freshw | 0.224 | 0.244 | 0.263 | 0.008 | | | 0.0004 | | | 0.038* |

5. Discussion

5.1 Sexual dimorphism

The data presented in this study show a strong sexual dimorphism in all five populations, regardless which ecotype they belong to. Differences between the sexes in specific traits have previously been reported in marine, anadromous and freshwater populations (Kitano *et al.*, 2007; Spoljaric and Reimchen, 2008; Aguirre and Akinpelu, 2010).

The sexual dimorphism in morphological traits can be the expression of shared and sex-specific components of variation, resulting in a complex, which is hard to disentangle (Hendry *et al.*, 2006). In natural populations of three-spined sticklebacks sexual dimorphism evolves despite a strong genetic between-sex correlation. Further it is not possible to predict the patterns of sexual dimorphism in wild populations from the genetic correlations in the ancestral population. Common-garden experiments show that sexual dimorphism in some traits has a genetic basis since all specimens are bred in the same habitat (Kitano *et al.*, 2007; Leinonen *et al.*, 2011a). But natural selection is also expected to influence the evolution of sexual dimorphism (Reimchen and Nosil, 2001; 2004). When sexual dimorphism actually develops during ontogeny in three-spined sticklebacks is not clearly determined. Kitano *et al.* (2007) found sexual dimorphism after the specimens started breeding, while Leinonen *et al.* (2011a) reported it already before the onset of breeding.

In this study a large number of changes between males and females occur in the head size and the head structures. The cs of the head and the length of the head are larger in the males. The cs of the eye is only larger when calculated relative to the size of the trunk, not relative to the size of the head, indicating that its size expands by the same factor as the head in comparison to the trunk. Further the structures in the mouth region (mouth, snout and jaw) are enlarged in the males. Similar results have been found in previous studies (e.g. Reimchen *et al.*, 1985; Caldecutt and Adams, 1998; Kristjansson *et al.*, 2002a; Kitano *et al.*, 2007). The enlarged head structures, especially in the mouth region, point towards a benthic orientation of the males referring to the feeding. The enlarged head proportions can be favourable when feeding an macrobenthos, because it maximizes the buccal volume and suction velocity (Caldecutt and Adams, 1998). Further Reimchen and Nosil (2001) found mostly benthic food items in the males' stomach confirming the suggestion that males

inhabit benthic niches. They also found differences in parasitism between males and females due to the variation in dietary niches, resulting in the different exposure to primary hosts. The use of the benthic niche is further explainable by the reproductive role of the males, since they build their nests in near-ground vegetation, transporting nesting material with the mouth and spend a long time period in this habitat protecting the nest and the juveniles (Wootton, 1984; Kristjansson *et al.*, 2002b; Reimchen and Nosil, 2004; Reimchen *et al.*, 2008; Aguirre and Akinpelu, 2010). The long snout is typical for male three-spined sticklebacks, but untypical for the adaptation to benthic habitats (e.g. Kitano *et al.*, 2007; Aguirre and Akinpelu, 2010). The experiments by Wund *et al.* (2008) showed that a benthic diet leads to specimens with a short snout. Also Caldecutt and Adams (1998) found short snouts in the benthic specimens of their wild-caught samples.

Focusing on the trunk, the males are deeper bodied with a short and deep caudal peduncle. This fits to the benthic orientation mentioned above, because it has been shown that these trait characteristics are favourable for rapid acceleration and manoeuvrability in dense vegetation (Webb, 1982; Taylor and McPhail, 1986; Hart and Gill, 1994; Walker, 1997).

The females, by contrast, are more limnetic, both with respect to the head structures and size as well as the overall body shape. Smaller heads are often found in female three-spined sticklebacks (e.g. Kitano *et al.*, 2007; Aguirre *et al.*, 2008; Aguirre and Akinpelu, 2010). The small mouth might lead to higher capture success in open waters (Schluter, 1993), which is often interpreted as an adaptation to a limnetic orientation. But the shorter snout is a disadvantage while feeding on limnetic prey as zooplankton (Walker, 1997). The overall body shape of the females is streamlined with a long and narrow caudal peduncle. This reduces the drag during steady swimming in open waters (Walker, 1997; Reimchen and Nosil, 2001). The results point toward the typical distribution in three-spined sticklebacks with more benthic males and limnetic females. The use of different spatial and dietary niches by the two sexes might be the result of avoiding intraspecific competition (Reimchen and Nosil, 2001). Contrasting findings were for example published by Kristjansson *et al.* (2002a), describing the males as more limnetic orientated than the females. Of course it must be taken into account that these results are pooled over 5 populations and 3 ecotypes. Therefore predictions on the distribution of the sexes into different ecological niches in specific populations are not possible.

The distance between the second and third dorsal spine is clearly larger in females. Spoljaric and Reimchen (2008) found also a difference in the distance between dorsal spines in females, but not between the second and third. Instead they found the distance between the first and the second dorsal spine enlarged in the females. This is one of the many examples of the high variation occurring in the sexual dimorphism in three-spined sticklebacks, which can be population specific despite the known genetic determination.

The anal fin is longer in males than in females, which was for example also found in a study of Spoljaric and Reimchen (2008). Walker (1997) predicted that specimens with a high risk of predation by vertebrate piscivores should have long and caudally placed median fins. Since the specimens from all populations are pooled for the analyses of the sexual dimorphism the population specific exposure to the different predator regimes cannot be classified properly. From the sampling site data it is known that at least the two populations from the fishponds (RJF and UPF) may be under a high predation pressure by piscivorous fish. Another suggestion refers to the reproductive role of the males and predicts an advantage due to the large anal fins in males during egg fanning, since it could give more stability in the trunk (Wootton, 1984; Foster, 1994; Walker, 1997). Since the anal fin is significantly longer although the populations have been pooled, it could be a trait occurring in all of the examined populations and thus the general explanation based on the reproductive role is likely.

Furthermore this study reveals differences in the pectoral girdle, both in position and size of the pectoral fin as well as in shape and size of the ectocoracoid. This can have different ecological reasons. On the one hand, Walker (1997) suggests that the size of the pectoral fin has an influence on the turning abilities important for predator avoidance, especially in dense vegetation. This would fit to the benthic orientation of the males, already found in the overall body shape and the head structures. On the other hand the reproductive role of the males is again an important factor. The males are fanning the eggs with the pectoral fins to ensure a good oxygen supply for the eggs, where a larger fin is favourable (Wootton, 1984; Whoriskey and FitzGerald, 1994; Walker, 1997). This is confirmed by experimental studies, showing a reduced paternal quality in males with reduced pectoral fin size (Bakker and Mundwiler, 1999; Künzler and Bakker, 2000). Additionally the pectoral fin is in a more posterior position in the males, which was also reported by Kitano *et al.* (2007).

For future analyses it would be interesting to examine the sexual dimorphism within each population or ecotype, to determine whether the sexual dimorphism is stronger developed in one of them.

5.2 Ecotype differentiation

First I focus on the differences between the marine and the anadromous ecotype. The marine ecotype is described as morphologically highly conserved whereas the anadromous ecotype is a more derived form (Bell and Foster, 1994b; Walker, 1997). In the literature these two ecotypes are alternately referred to as marine, anadromous or oceanic three-spined sticklebacks. The terminology is often used imprecisely, but the examined ecotype can often be identified from the sampling site. This study shows that there are differences between these two ecotypes in *G. aculeatus* and they should therefore not be treated as the same, especially in comparative studies with freshwater populations because already the anadromous ecotype is a derived form and not the most ancestral one (Wootton, 1984; 2009; Bell and Foster, 1994a; Paepke, 2002). The mean shapes of the marine and the anadromous ecotype in this study are significantly different (Tab. 9). Only the high Bonferroni-correction factor due to the detailed examination of specific morphological traits masks this fact.

The marine ecotype was in this study found to be deeper bodied, with a short and thick caudal peduncle, a longer dorsal fin and a larger ectocoracoid. The anadromous specimens are more streamlined with a long and narrow caudal peduncle. This points towards an adaptation to long swimming distances in open waters in the anadromous ecotype, fitting the migrating life cycle (Taylor and McPhail, 1986; Walker, 1997). Also the marine population (SPK) has to migrate from the Sylt-Rømø bight into the open waters of the North Sea. This is caused by the low winter temperatures typical in the temperate zone in the shallow coastal waters, the February long-term mean in List, Sylt is 2.3 °C, and the high current velocity in the deeper tidal creeks since three-spined sticklebacks prefer still to slow-moving water (e.g. Wootton, 1984; Guderley, 1994; Diederich *et al.*, 2005). But these migrating distances seem to be shorter than the ones of the anadromous specimens which have to migrate further into upcountry freshwater habitats for spawning and not only into the coastal areas. Further longer dorsal and anal fins are a more efficient way of increasing the area of the caudal peduncle than increasing the peduncle depth itself, which is favourable in the predator

avoidance by acceleration movements (Walker, 1997). The marine ecotype has both, a deep caudal peduncle and an enlarged dorsal fin, which could indicate predator avoidance by burst swimming. The head size and structures, which are so dominant in the sexual dimorphism, show no differences between these two ecotypes, and could indicate similar feeding patterns. But this has to be analysed separately, for example by stomach content examinations.

In the literature the marine or anadromous populations are only compared with different freshwater populations but not with each other, therefore no comparable results are available. This study is a first glance on the differences between these two ecotypes and it would be desirable if more studies, also in other geographical regions, would follow.

Furthermore the results of this study show that the three anadromous populations are an intermediate state between the marine and the freshwater ecotype, when they are classified by their overall body shape and morphological traits. Note especially the shift in the deformation grids of the anadromous population towards the freshwater ecotype body shape when compared with the marine ecotype (Fig. 23), and towards the marine ecotype body shape when compared to the freshwater ecotype (Fig. 24). This confirms the nowadays established opinion that the marine, conserved ecotype in the three-spined stickleback is the most ancestral one from which the anadromous and further the very derived freshwater ecotype populations have evolved, as mentioned in chapter 2.2 (e.g. Münzing, 1959; Bell and Foster, 1994b; Walker, 1997; Paepke, 2002; Bell *et al.*, 2004; Colosimo *et al.*, 2005; Wootton, 2009).

The three anadromous populations migrate into different areas of the Danish Wadden Sea. The HSS population migrates into the Ho-bight, the RJF population migrates into the bight between Mandø and Rømø and the BKD population migrates into the Sylt-Rømø bight. These three populations differ from each other in body shape, indicating several individual anadromous populations in the North Sea and not one large anadromous panmictic North Sea population. For the analyses, the three anadromous populations have been pooled to determine the differences between the ecotypes, and not only single populations.

Comparing the anadromous ecotype with the freshwater ecotype, the anadromous one is deeper bodied with a shorter and thicker caudal peduncle. Further the ectocoracoid is

enlarged in the anadromous specimens with a change in the angle of the caudal edge, influencing the position of the ventral origin of the pectoral fin. The only significant difference in the head structures is found in the eye, which is smaller in the anadromous ecotype. The findings in this study on body depth and caudal peduncle height are contrary to e.g. Taylor and McPhail (1986), Spoljaric and Reimchen (2007; 2011). The other findings in this comparison (length of the caudal peduncle, size of the eye and size of the ectocoracoid) are confirmed by previous studies (Leinonen *et al.*, 2006; e.g. Spoljaric and Reimchen, 2007; Aguirre, 2009). It is surprising that the freshwater ecotype is more streamlined than the anadromous ecotype, since the latter should have longer swimming distances due to their migrating life cycle. The limnetic body shape of the freshwater specimens can be caused by the specific shape of the fishpond, with a short shallow shore area and a steep slope caused by the gravel dredging. Therefore the benthic areas in this pond are limited and the open water habitat is larger than usually in ponds of this size. Similar results were reported by Walker and Bell (2000), who found a reduction in body depth under specific conditions (low relative littoral area) in freshwater habitats. The reduction in the size of the ectocoracoid cannot be explained by the reproductive behaviour, since only males were investigated for the ecotype differentiation. But there are two other possible explanations. The first one suggests that the decrease in the ectocoracoid size is due to a reduction of the entire bony body armour as often observed in freshwater three-spined sticklebacks. In this case it could be as a response to a specific loci variation linked to a Quantitative trait loci (QTL) in the major gene Ectodysplasin (*Eda*) which strongly influences body shape and bony structures such as the lateral plates (Colosimo *et al.*, 2005; Albert *et al.*, 2008; Aguirre and Bell, 2012). The second explanation focuses on the locomotion. *G. aculeatus* has a labriform locomotion, using exclusively the pectoral fins for many locomotor behaviours, such as low-acceleration swimming, many high-accelerations movements and hovering (Taylor and McPhail, 1986; Walker and Bell, 2000). The muscle mainly active during the power stroke is the deep adductor muscle, which arises from the ectocoracoid in *G. aculeatus* (Westneat and Walker, 1997; Walker and Bell, 2000). Walker and Bell (2000) suggest that freshwater three-spined sticklebacks have lower activity levels than marine or anadromous specimens. Further freshwater three-spined sticklebacks are often smaller in size, resulting in lower body mass and leading to lower size-specific power requirements to the pectoral fin musculature.

Therefore it is possible that the reduction in the ectocoracoid size is the result of selection for smaller pectoral fin muscles which then also need smaller insertions.

Aguirre and Bell (2012) are describing a former anadromous population introduced into a freshwater lake 20 years ago. Their findings are very interesting, since the freshwater population in this study (UPF) is a similar case and gives further insights into this topic. The lakes of these two studies are about the same size. The fishpond where the UPF population was sampled has been established as an artificial recreational lake about 20 years ago. The changes in body shape observed on the UPF population and the population in the study of Aguirre and Bell (2012) are very much alike. They found that the specimens late in the time series showed an elongated caudal peduncle, a decrease in body depth and the length of the ectocoracoid and more posterior dorsal spines. This study is already another good example for parallel evolution under similar conditions, but future studies are necessary to determine how general these findings are in freshwater populations recently founded by anadromous specimens.

The third comparison is between the conserved marine ecotype and the derived freshwater ecotype. As expected, this comparison revealed the most eye-catching differences in body shape. The specimens of the marine ecotype are clearly deeper bodied with a shorter and deeper caudal peduncle. The changes in the ectocoracoid already mentioned above are here extremely developed, leading to a more anterior position of the ventral origin of the pectoral fin in the freshwater ecotype, resulting in a different position of the fin in total. There are no differences in the head structures besides the enlarged eye in the freshwater ecotype and the grids show a trend of a slightly different position of the mouth. The results of this study are congruent with the findings of Leinonen *et al.* (2006), who also described the marine ecotype as deeper bodied, with a short caudal peduncle and smaller eye than the freshwater population. This is partially contrary to Klepaker (1993), who also found smaller eyes and a short caudal peduncle in the marine three-spined sticklebacks but they had more elongated bodies than the specimens of the freshwater populations. The short caudal peduncle seems to be a general pattern in the marine ecotype.

Both the comparisons of the freshwater ecotype with the marine and the anadromous ecotype indicate that the freshwater population (UPF) analysed in this study is exceptional. It has characteristics of both the limnetic body shape (streamlined body with a long and

narrow caudal peduncle) and the benthic body shape (large eye). This freshwater population was assumedly founded in the end of the 1980s from an anadromous population, because at this time the gravel dredging stopped and there are various creeks belonging to the Vidå river system in the area inhabited by anadromous three-spined sticklebacks. It is not known if the introduction occurred by only one single event or if there were multiple introduction events. But a frequent introduction into the fishpond by stocking with trouts can be excluded. A founder effect could possibly cause this unusual body shape in the freshwater population.

In summary, this study showed a continuous trend from the marine to the anadromous and the freshwater population in the following traits: the size of the eye is increasing, the body depth and the size of the ectocoracoid are decreasing and the ventral origin of the pectoral fin is shifted in a more anterior position. Further it has been shown that there is a difference between the marine and the anadromous ecotype of *G. aculeatus*. This difference can confound the result of comparative studies between either marine or anadromous populations with freshwater populations. Therefore these two ecotypes should be treated separately. Future studies on these two ecotypes are necessary to determine the exact differences, also for other geographical regions. Moreover the sexual dimorphism is a huge factor in body shape variation which has to be taken into account whenever body shape analyses of three-spined sticklebacks are done.

6. References

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Fig.1. Wootton, R. J. (1984). *A functional biology of stickleback*. London: Croom Helm.

Fig.2. modified after Paepke, H.-J. (2002). *Gasterosteus aculeatus* Linnaeus, 1758. In *The freshwater fishes of Europe*, pp. 209-256. Wiebelsheim, Germany: AULA-Verlag GmbH Wiebelsheim, Verlag für Wissenschaft und Forschung.

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Fig. 15. Mitteroecker, P. & Gunz, P. (2009). Advances in Geometric Morphometrics. *Evolutionary Biology* **36**, 235-247.

Acknowledgements

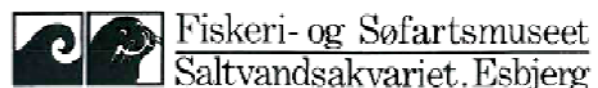
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